IMMUNOGENICITY AND PROTECTIVE EFFICACY EVALUATION OF CANARYPOXVIRUS(ALVAC)-BASED FIV VACCINES IN CATS

By

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
BM	Bone marrow
BSA	Bovine serum albumin
CTL	Cytotoxic T-lymphocyte
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
HIV	Human immunodeficiency virus
LN	Lymph node
MHC	Major histocompatibility complex
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcriptase
SDS	Sodium dodecyl sulfate
SIV	Simian immunodeficiency virus

TAE	Tris, EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
THY	Thymus
Tris	Tris(hydroxymethyl)aminomethan
VN	viral neutralizing
WB	western blot

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The infection of cats with the feline immunodeficiency virus (FIV) provides a valuable animal model for the assessment of therapeutic and vaccine strategies against human immunodeficiency virus (HIV) in man. A promising candidate vaccine tested presently in human volunteers is the recombinant canarypoxvirus vector ALVAC. This vector has also been used with some success against HIV infection in the macaque and chimpanzee models. Herein, the efficacy of ALVAC-based vaccines was evaluated against experimental FIV infection in cats. Two approaches were evaluated which included ALVAC-based FIV vaccines alone or in combination with conventional inactivated FIV-infected cell vaccine (ICV).

Immunization schemes employing ALVAC-FIV recombinants alone effectively induced FIV-specific cytotoxic T-cell responses. However, these schemes failed to induce humoral responses including viral neutralizing antibody responses. Immunization schemes employing ALVAC-FIV recombinants combined with conventional inactivated FIV-infected cell vaccine induced FIV-specific cytotoxic T-cell responses and FIV-specific humoral responses but lacked detectable viral neutralizing antibody responses.

Cats immunized with the ALVAC recombinants encoding the FIV core (Gag) protein were protected from challenge exposure with 50 ID_{50} FIV Petaluma, a subtype A FIV isolate highly related to the Ville franche isolate basis of the ALVAC-FIV vaccine. In contrast, ALVAC recombinants expressing the FIV envelope alone or both the FIV envelope and Gag proteins failed to induce such protection. Cats immunized with ALVAC-FIV recombinants and boosted with ICV were also protected from a FIV Petaluma challenge exposure. In addition, these cats were partially protected from challenge with 75 ID_{50} FIV Bangston (subtype B), a distinctly heterologous isolate, given eight months after the initial challenge without any intervening booster.

In conclusion, vaccine protocols employing recombinant ALVAC-based FIV vaccines alone or in combination with conventional inactivated FIV-infected cell vaccine can prevent establishment of FIV infection in cats. This immunity may even protect or delay infection with FIV isolates of other subtypes than those used to generate the vaccine. It remains unclear as to what constituted protective immunity in the protected animals. The obtained data suggest a role for cell-mediated responses. However, a role for FIV specific humoral responses including viral neutralizing antibody responses can not be excluded.

CHAPTER I
LENTIVIRUSES AND VACCINE DEVELOPMENT

Introduction

Lentiviruses comprise a group of viruses known to cause life-long chronic infections in a number of species (Table 1.1). The most prominent member of this group is the human immunodeficiency virus (HIV), the causative agent of an acquired immunodeficiency syndrome (AIDS) in man. HIV was first isolated from young male homosexual patients in 1983 (Barre-Sinousi et al. 1983; Gallo et al. 1984). These patients presented with a high incidence of a pneumonia caused by *Pneumocystis carinii* thus far only known to cause disease in immuno-compromised people and a rare cancer, *Kaposi sarcoma* (Ammann et al. 1983; Gyorkey et al. 1984; Selik et al. 1984; Weissler 1990). More important, it was noticed that the numbers of circulating lymphocytes, in particular those of the $CD4^+$ T-helper phenotype, were severely reduced in these patients, a phenomenon which is now considered one of the main hallmarks of HIV infection in man (Huet et al. 1990). Many years prior to the discovery of HIV, a number of viruses, much later classified as lentiviruses, had been described as pathogens in animals. This included the equine infectious anemia virus (EIAV) which was identified in 1904 as the causative agent of a disease in horses characterized by recurrent episodes of fever and hemolytic anemia (Vallee and Carre 1904). This also included the maedi-visna virus (MVV) which was first isolated from sheep in Iceland that presented with severe chronic pneumonia (maedi), wasting and paralysis (visna) (Gislason 1947; Narayan et al. 1977). Based on the long incubation period and the fact this virus could manifest its effects over a long period,

MVV was named a lenti- (slow) virus (Sigurdsson 1954). This virus has since become the prototype of the lentivirus genus. In 1974, a virus similar to MVV was identified in goats, the caprine arthritis encephalitis virus (CAEV). CAEV infection presents as chronic inflammation of the joints in adult goats and progressive encephalopathy in younger goats (Clements et al. 1980; Cork 1974).

Thus, several animal lentiviruses had been long known before HIV. However, it was the discovery of HIV that resulted in an increased interest in these viruses and led to the search and isolation of lentiviruses in other species. For example, a number of lentiviruses were isolated from monkeys. These viruses have collectively been named the simian immunodeficiency viruses (SIV) and are specified by the particular monkey species they have been isolated from (Table 1.1) (Huet et al. 1990; Kanki et al. 1985; Ohta et al. 1988; Tjusimoto et al. 1988; Hirsch et al. 1993; Peeters et al. 1992; Fultz et al. 1986). In contrast to HIV infection of man, SIV infection of natural host African monkey species, is relatively nonpathogenic. However, the exception to this is SIV infection in Asian macaques (Daniel et al 1985; Benvisti et al. 1986). Infected Asian macaques develop an acquired immunodeficiency syndrome similar to that of HIV infected individuals (Murphey-Corb et al. 1986). Interestingly, most of the nonpathogenic SIV strains have been isolated from monkeys in the wild. SIV_{mac}, however, has only been isolated from macaques held in captivity and has never been isolated from this species in its natural habitat. Since these monkeys are of Asian origin whereas other SIV infected monkeys are of African origin it has been suggested that this virus was transmitted to macaques during captivity from an African monkey species, most likely the mangebeys (Murphey-Corb et al. 1986).

The second human lentivirus, now known as HIV-2, was first isolated in 1986 (Clavel et al. 1986). HIV-2 is predominantly found in West-African prostitutes and also causes AIDS although milder in its pathogenesis as marked by a longer incubation period and a lower rate of transmission (Marlink et al. 1994). Much like SIV infection of Asian

macaques, the emergence of HIV in humans is thought to be caused by cross-species transmission. This is supported by the fact that HIV-2 genetically closely resembles the SIV_{smm}- and SIV_{mac}-isolates, and HIV-1 more closely resembles the SIV_{cpz}-isolate (Hirsch and Johnson 1994; Franchini et al. 1987).

Table 1.1 The Lentiviruses

Virus	subtypes	species	Clinical signs of disease
EIAV		horse	anaemia fever weight loss
Maedi-visna		sheep	encephalomyelitis wasting pneumonia
CAEV		goats	arthritis encephalomyelitis wasting
BIV		cows	lymphadenopathy lymphocytosis wasting
FIV		cats	immunodeficiency opportunistic infections neurological disorders
HIV	HIV-1	human	immunodeficiency lymphadenopathy neurological syndrome opportunistic infections
	HIV-2	human	immunodeficiency lymphadenopathy opportunistic infections
SIV	SIV_{mac}	macaques	immunodeficiency neurological disease
	SIV_{smm}	sooty mangabey	
	SIV_{agm}	African green monkey	
	SIV_{mnd}	mandrill	no obvious clinical signs
	SIV_{syk}	Sykes monkey	of disease
	SIV_{cpz}	chimpanzee	

In addition to the primate lentiviruses, a virus similar in morphology and genetic composition, the bovine immunodeficiency virus (BIV), was isolated from cows in 1985. Although, the pathogenesis of BIV is poorly defined, infection in calves has been associated with lymphocytosis and lymphadenopathy (Gonda et al. 1987, 1994).

The feline homologue of HIV, feline immunodeficiency virus (FIV) was first isolated in 1986 from a cattery in California (Pedersen et al. 1987). In this cattery, several cats presented with a loss in immune function after the introduction of a sentinel cat. The loss of immune function could not be linked to feline leukemia virus (FeLV), another member of the Retroviridae, already known to cause immunosupression in cats (Jarret et al. 1964). This led to the discovery of a novel retrovirus that differed from FeLV and more closely resembled HIV in morphology and the Mg^{2+}- rather than $Mn2^{+}$-dependence of its reverse transcriptase (Yamamoto et al. 1988a). Subsequent genetic analysis demonstrated that this virus belonged to the lentivirus family.

The seven known members of the lentivirus family as listed above are commonly divided into two groups based on differences in cell tropism and disease manifestation. Those affecting the ungulate species EIAV, MVV, and CAEV are predominantly macrophage-tropic and cause immune-mediated diseases that target specific organs. Those affecting primates, HIV and SIV, are tropic for lymphocytes and macrophages and cause a major loss of immune function that results in an increased susceptibility to opportunistic pathogens. FIV resembles the primate viruses in cell tropism and disease manifestation but is genetically more closely related to the nonprimate lentiviruses (EIAV and MVV) (Olmsted et al. 1989b).

Common to all lentiviruses is the long incubation period, the ability to affect multiple organs, and most importantly the persistence in the face of host-immune responses. The ability to escape from the host immunity is in part explained by a high mutation rate of the lentiviral genome resulting in continuous antigenic variation (Rigby et

al. 1993). As such, lentiviruses present a major challenge to the development of therapeutic strategies and vaccines.

The Feline Immunodeficiency Virus

The FIV virion, like other members of the Retroviridae, is enveloped and approximately 100-125nm in diameter (Pedersen et al. 1987). A electron micrograph of a mature lentivirion is depicted in Figure 1.1. The FIV core, composed of the capsid protein (CA, p24), has the physical appearance of a cone, typical of all lentiviruses. The core encloses the viral genome which consists of two identical single stranded RNA molecules of approximately 9kb (Elder et al. 1993). Associated with the viral genome is a small nucleocapsid protein (NC, p8), which is thought to play a role in viral assembly and disassembly (Elder et al. 1993; Aldovini and Young 1990). Also packaged within the virion are the enzymes essential for replication, the reverse transcriptase (RT), the integrase (IN) and the deoxyuridine triphosphatase (dUTPase; DU)(Elder et al. 1993). The RT is responsible for transcription of the viral RNA genome into DNA. The integrase functions as an endonuclease which cuts the cellular genome to allow integration of the provirus. The dUTPase is unique to the nonprimate viruses and its function in the FIV life cycle still remains unknown. However, it is speculated that the dUTPase is required for replication of FIV in nondividing macrophages or resting T-lymphocytes (Miyazawa et al. 1993). Additionally, it has been observed that lack of functional FIV dUTPase results in an increased mutation frequency of FIV propagated in macrophages (Lerner et al. 1995). Surrounding the viral core is a matrix protein layer (MA, p15) which is closely associated with the viral envelope. The viral envelope is derived from the cellular membrane by budding of the viral particles and consists of lipids, inserted viral proteins and cellular proteins (Gelderblom et al. 1987). The virally derived envelope glycoprotein (Env) is composed of two subunits; the transmembrane protein (TM, gp40) and the surface protein

(SU, gp120). The TM protein protrudes through the viral membrane and noncovalently anchors the outer SU protein, which appears as a knob-like structure on the viral surface.

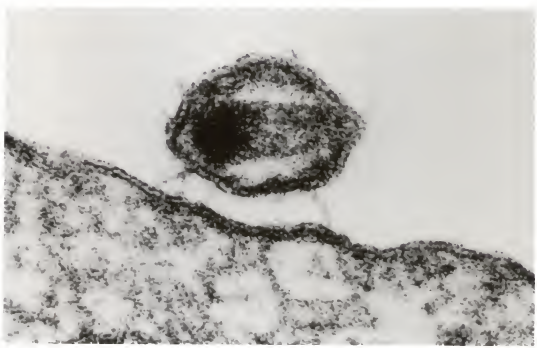


Figure 1.1 Electron micrograph of FIV x70,000.

FIV genomic organization and regulation of gene expression

Lentiviral genomes are among the smallest of the known viruses and are more complex in their genomic organization than other members of the retrovirus family. The FIV genome is a positive stranded polyadenylated RNA of approximately 9kb. It contains three major open reading frames (ORFs) encoding the structural and enzymatic proteins (Env,Gag and Pol) necessary for the viral life cycle (Olmsted et al. 1989a, 1989b;

Miyazawa 1993). These genes are organized in the order of 5'-gag-pol-env-3', typical of all replication-competent retroviruses. In addition, lentiviruses contain several small open reading frames (ORFs) that encode for auxiliary proteins. At least 4 ORFs have been identified for FIV which may encode for regulatory proteins similar to those described for the primate lentiviruses (Miyazawa et al. 1993; Olmsted et al. 1989b). Flanking the proviral genome are long terminal repeat (LTR) regions which are crucial for integration of the proviral DNA into the cellular genome. These regions also contain enhancer and promoter elements which are required for efficient transcription of the retroviral genome, as well as, a polyadenylation signal sequence (Phillips et al. 1992; Talbott et al. 1989).

Transcription of the integrated proviral DNA genome initiates at the 5'LTR to generate full length viral mRNA transcripts (Miyazawa et al. 1993). Initially, these transcripts will undergo multiple splicing and give rise to small mRNAs that encode for regulatory proteins such as Tat and Rev. The Tat protein facilitates gene expression from the 5'LTR. Tat activity is essential for replication of all primate lentiviruses. In contrast, Tat is not essential for replication of FIV in T-lymphoblast cells (Sparger et al. 1992; Miyazawa et al. 1993). The Rev protein, encoded by all members of the lentiviridae, is responsible for shifting viral gene expression from early regulatory proteins to that of structural and enzymatic proteins. It accomplishes this by binding to the Rev-responsive element (RRE) in the *env* coding region of single spliced and unspliced full length mRNAs. In doing so, it promotes the stability and transport of incompletely spliced mRNAs from the nucleus to the cytoplasm (Phillips et al. 1992; Cochrane et al. 1990; Hammarskjold et al. 1989; Stephens et al. 1992). The single spliced messages give rise to the Env precursor protein which is cleaved into the transmembrane glycoprotein (TM) and the outer surface glycoprotein (SU) by cellular proteases (Stephens et al. 1991; Talbott et al. 1989).

The unspliced full length mRNA serves as both a template for the Gag and Gag-Pol proteins and as genome that is packaged into the viral core. Regular translation of full length message gives rise to the Gag precursor protein which is cleaved into the mature

capsid (CA), matrix (MA) and nucleocore (NC) proteins by the viral protease encoded within the *pol* gene (Elder et al. 1993). Expression of the Gag-Pol precursor protein from the unspliced full length mRNA is accomplished by a ribosomal frameshift that prevents termination of translation at the *gag* stop codon (Morikawa and Bishop 1992). The efficiency of this shift is about 5%, so that the production of Gag protein is 20 fold higher than that of Gag-Pol polyprotein. The Gag-Pol precursor protein is proteolytically processed into the viral protease (PR), the reverse transcriptase (RT), the deoxyuridine triphosphatase (DU) and the integrase (IN) proteins (Elder et al. 1993).

FIV replication

The first step in the replication of FIV is the attachment of the virus to the cell receptor. For HIV, the major receptor has been identified as the CD4 molecule, present on T-helper lymphocytes (Dagleish et al. 1984; Klatzman et al. 1984). In addition, the CD26 molecule and the recently described fusin and CKR-5 molecules have been reported to play a role in HIV attachment and fusion (Callebaut et al. 1993; Feng et al. 1996; Alkhatib 1996). The fusin is thought to act as a coreceptor for T cell line-tropic HIV strains whereas the CKR-5 is thought to act as a coreceptor for macrophage-tropic HIV strains (Feng et al. 1996; Alkhatib 1996). The target receptor(s) for FIV, however, is still unknown. Nonlymphoid feline cell lines transfected with cDNA encoding the feline CD4 (fCD4) protein failed to support productive infection indicating that the fCD4 alone is not sufficient (Norimine et al. 1993). Others have proposed a putative role for a receptor homologous to the human CD9 molecule that is expressed on both haematopoietic and nonhaematopoietic cells (Willet et al. 1994; Hosie et al. 1993; Boucheix and Beiot 1988). Anti-CD9 antibodies effectively block replication of FIV infection on lymphoid cells and ectopic expression of CD9 on feline lymphoma cells causes an enhancement of viral infection with cell culture

adapted FIV strains. However, ectopic expression of CD9 on these cells does not seem to render these cells more susceptible infection with primary FIV isolates (Willet et al. 1996).

Following attachment, the FIV enters the cell either by receptor mediated endocytosis or by fusion of the viral envelope with the cellular membrane. Upon entry the viral genome is released into the cellular cytoplasm. Subsequent, the viral RNA genome is transcribed into double-stranded proviral DNA by the viral reverse transcriptase packaged within the virion. Similar to HIV, initiation of first strand cDNA synthesis is primed by cellular $tRNA^{lys}$ (Olmsted et al. 1989b; Talbott et al. 1989). Integration of the provirus into the host cellular genome is facilitated by the viral Integrase and occurs at random sites. The integrated provirus can stay quiescent or give rise to progeny viral particles. During productive infection, the proviral DNA is transcribed into viral mRNAs by cellular RNA polymerase II. These mRNAs are translated into the Gag precursor (p55), the Gag-Pol precursor (p160) and the Env precursor proteins (gp160). The Gag precursor is further processed by proteolysis to give rise to the capsid (CA), matrix (MA), and nucleocapsid (NC) proteins. Proteolysis is mediated by the viral protease which also facilitates its own cleavage (Elder et al. 1993). Similar to HIV, the FIV matrix protein is myristolated. Myristolation is the attachment of a C14 fatty acid and is required for proper targeting of the MA protein to the cellular membrane (Elder et al. 1993). The Env precursor protein is cleaved into the mature SU and TM proteins by cellular proteases and further processed by glycosylation. For FIV, 22 possible N-linked glycosylation sites have been identified; 18 in the SU and 4 in the TM protein (Stephens et al. 1991; Elder et al. 1993). The final step in the FIV replication cycle involves the assembly of the virions and their release from the cell by budding.

FIV cell tropism

FIV has a broad cell tropism and infects cells of both lymphoid and monocyte/macrophage origins. In contrast to HIV, which is thought to primarily replicate in $CD4^+$ T-lymphocytes and not in $CD8^+$ T-lymphocytes, FIV productively infects both $CD4^+$ and $CD8^+$ T-lymphocytes (Brown et al. 1991). Additionally, FIV has been shown to replicate in B-cells, thus further supporting the view that the feline CD4 receptor is not the primary cell receptor for FIV, as it is for HIV (English et al. 1993; Norimine et al. 1993). The macrophage/monocyte cell types supporting FIV replication include peritoneal macrophages, Kupffer cells in the liver, microglial cells, astrocytes, and endothelial cells in the central nervous system (Steffan et al. 1994; Dow et al. 1990; Martin et al. 1995; Brunner and Pederson 1989). Furthermore, a number of FIV isolates have been shown to infect cells of nonlymphoid origin including Crandell feline kidney cells (CrFK) and feline tongue cells (Fc3Tg) (Yamamoto et al. 1988a).

FIV epidemiology and pathogenesis

FIV has been isolated from cats worldwide. The virus infects domestic cats (*Felis catus*) and is species specific (Yamamoto et al. 1988b, 1989). FIV-related viruses have been isolated from several wild felids including the African lion (*Panthera leo*), and the Pallas cat (*Felis manul*) (Barr et al. 1989, 1995; Poli et al. 1995; Brown et al. 1994). Furthermore, serologic surveys in African and Asian lions revealed that the serum of the majority of these animals reacted positively with FIV (Brown et al. 1994).

The prevalence of FIV varies throughout the world. In North America the average incidence is estimated at 1.4% in healthy animals and 7.4% in diseased cats (Yamamoto et al. 1989; Shelton et al. 1990). The incidence of infections is the highest in free roaming, outdoor male cats. Since FIV is shed in the saliva, the major route of transmission is most

likely due to biting between male cats as part of territorial behavior (Yamamoto et al. 1989). In addition to saliva, FIV can be recovered from blood, serum, plasma, and cerebrospinal fluid of infected cats (Pedersen et al. 1987; Dow et al. 1990). Horizontal transmission through contact alone appears to be inefficient (Pedersen et al. 1987; Yamamoto et al. 1988b). Vertical transmission *in utero* or postpartum via the milk has been reported and was found to occur most frequently in queens that became viremic during pregnancy (Callanan et al. 1991; Wasmoen et al. 1992; O'Neil et al. 1996). Although high rate of perinatal transmission has been reported for queens that had been infected with a highly pathogenic FIV strain 4 to 30 months prior to conception (Ueland and Nesse 1992; O'Neil et al. 1996).

Similar to HIV, five clinical stages can be defined for FIV infection in cats. The acute viremic phase, 2 to 4 weeks after infection, is characterized by fever, neutropenia, and generalized lymphadenopathy. These symptoms vary in duration and severity between individual cats and are mostly recognized in experimentally infected cats but rarely in naturally infected cats (Yamamoto et al. 1988b, 1989). Once a full immune response is established and most of the virus is cleared from the plasma, a period of months to years follows with no obvious clinical signs of disease, defined as the asymptomatic phase. However, during this period changes in lymphocyte counts, such as a decrease in $CD4^+$ lymphocytes and CD4/CD8 ratios, takes place (Ackley et al. 1990). This period is followed by a phase equivalent to that of AIDS related complex (ARC) in men. Cats with ARC often present with chronic illness such as stomatitis/gingivitis, lower urinary tract infections, skin disorders and diarrhea (Yamamoto et al. 1989). Finally, cats may develop a stage similar to that of AIDS in men, characterized by severe lymphoid depletion, weight loss and opportunistic infections. Opportunistic pathogens reported in cats suffering from AIDS include toxoplasmosis, cryptococcoses, candidiasis, mycobacteriosis, feline calici- and herpes virus (Lapin et al. 1989; Knowles et al. 1989; Ishida and Tomoda 1989). At this stage, $CD4^+$ T-cell counts have dropped dramatically and CD4:CD8 ratios are inversed

(Ackley et al. 1990). Other immunologic abnormalities reported include hypergammaglobulinemia and reduced T-cell responses to T-independent antigens (Ackley et al. 1990; Torten et al. 1991). The occurrence of neurological abnormalities as seen in AIDS patients has only been reported in a small percentage of cats infected with neurotropic isolates of FIV (Podell et al. 1993; Dow et al. 1990).

FIV as an animal model for HIV

With the emergence of the HIV pandemic and an estimated 12 million infected people, animal models to study antiviral drugs and vaccine strategies have become very important. The search for an appropriate animal model for HIV-1 infection in man has, however, been complicated by the host specificity of HIV-1.

To date, HIV-1 has only been shown to infect three other species; pig-tailed macaques, gibbons, and chimpanzees (Agey et al. 1992; Fultz et al. 1986). These animals become viremic and mount specific antibody responses similar to HIV infected humans. However, infection does not result in depletion of $CD4^+$ T-lymphocytes, immunosuppression, or opportunistic infections. Furthermore, chimpanzees are an endangered species, therefore limiting the availability of these animals for research purposes. Consequently, the infection of macaques with SIV has become the most prevalent animal model used. Both the SIV_{mac}- and SIV_{smm}- isolates have been shown to cause an AIDS-like disease in rhesus-, cynomolgus-, and stumptail macaques (Murphey-Corb et al. 1986). Initial infection results in viremia and is characterized by fever, diarrhea and lymphadenopathy. Like HIV-1 in humans, monkeys in the end-stages of disease present with opportunistic infections and show decreased $CD4^+$ T-lymphocyte counts and inversed CD4:CD8 ratios. Additional promising models include the infection of baboons and pig-tailed macaques with specific isolates of HIV-2 (Barnett et al. 1994; Novembre et al. 1994). A large percentage of these monkeys become persistently infected and show

depletion of $CD4^+$ T-lymphocytes and susceptibility to opportunistic pathogens. In addition, several alternative models have been developed with some success. For example, transient HIV infection of SCID mice reconstituted with human lymphocytes or peripheral blood mononuclear cells (PBMC) and the infection of rabbits with HIV-1 (Reina et al. 1993; Mosier et al. 1991; Namikawa et al. 1988). These models, however, are highly artificial and the relevance to HIV pathogenesis in humans should be interpreted with caution.

The infection of FIV in domestic cats offers several advantages over the models discussed above. First, FIV is a natural pathogen of cats and the pathogenesis closely resembles that of HIV in man. Obvious advantages of the feline model include the availability and costs which allow the use of larger study groups. Especially relevant to vaccine development is the availability of a wide variety of FIV subtypes which is lacking in the SIV model. FIV isolates have been grouped into 4 subtypes (A-D) versus 7 for HIV (Sodora et al. 1994; Kakinuma et al. 1995; Rigby et al. 1993). This grouping is primarily based on antigenic diversity in the Env and Gag proteins. Hence, the FIV model provides a means to evaluate the protective efficacy of vaccine strategies against multiple subtypes and as such has implications to the development of multiple subtype HIV vaccines. Further, FIV like HIV replication is sensitive to antiviral drugs such as AZT and protease inhibitors (North et al. 1989, 1990). Thus, FIV infection in cats also provides a model to assess the efficacy of these and other newly developed drugs.

Vaccine development

Vaccine development initiated with the work of Dr. Edward Jenner in 1798. He observed that milkmaids which had recovered from cowpox did not contract the more virulent smallpox. Based on this observation he postulated that smallpox infection of man could be prevented by prior exposure to cowpox. He successfully proved this hypothesis

by demonstrating that a boy, injected with material from a cowpox pustula, failed to develop disease upon exposure to smallpox. This technique became known as vaccination (Jenner 1798).

Though the underlying mechanisms were not known at the time, it is now understood that success of vaccination lies in the ability of the immune system to generate long lasting immunity. This immunity is mediated by memory B and T lymphocytes which are capable of rapid anamnestic responses upon exposure to foreign antigens. Responses mediated by B cells include the production of specific antibodies that could prevent the entry of pathogens into host cells by interfering with microbial attachment or fusion upon attachment to host cells. These antibodies, in respect to viral pathogens, have been defined as viral neutralizing (VN) antibodies. Further, antibodies may directly destroy microbes by complement mediated lysis or promote phagocytosis by macrophages and natural killer cells through opsonization. T-lymphocyte responses include those mediated by T-helper cells and T-cytotoxic lymphocytes (CTLs). T-helper cells are of the $CD4^+$ phenotype and recognize exogenously produced antigens presented in the context of MHC-class II found predominantly on B-lymphocytes and macrophages. Upon recognition these cells produce interleukins to facilitate the activation of macrophages and maturation of B cells into antibody producing plasma cells. Cytotoxic T-lymphocytes of the $CD8^+$ phenotype function by the direct destruction of infected cells displaying foreign antigens in association with MHC-class I molecules. Class I MHC molecules are found on the majority of cells and present endogenously synthesized antigens. As such CTL responses are especially critical to the clearance of intracellular bacteria and virally infected cells. Together, humoral and cell mediated responses are capable of preventing the invasion of pathogens.

Several different types of vaccines have been developed. Most commonly used vaccines against viral pathogens are composed of live attenuated viruses, inactivated whole virus, or inactivated virus infected cells. The majority of these vaccines induce VN antibody responses and some, in particular attenuated live viruses, also induce cell-

mediated responses including CTL reponses. More recent developments in the vaccine field involve the use of viruses, bacteria or naked DNA as vaccine vectors. These vectors are genetically engineered to carry and express foreign genes encoding immunogens of pathogens. Upon inoculation of these vectors into the host, the inserted immunogen is expressed and presented to the host immune system. In fact, viral antigens encoded within these vectors are presented to the host immune system in a manner simulating natural infection. Furthermore, vector based vaccines are thought to be more effective in eliciting CTL responses than conventional inactivated vaccines. As such, vaccine strategies employing vector based vaccines may be especially useful against viral and intracellular bacterial pathogens.

To date, success of viral vaccines has been limited to a confined group of viruses. These viruses display constant antigenic specificity and consist of a single or limited number of serotypes. Furthermore, spontaneous recovery has usually been observed shortly after natural infection with these viruses. Lentiviruses, including FIV, do not fall into this category. Lentiviruses are subject to continuous antigenic variation and consists of many serotypes. For FIV a total of 4 subtypes have been defined based on genetic differences in the *env* and *gag* coding regions (Sodora et al. 1994; Kakinuma et al. 1995; Rigby et al. 1993). Spontaneous recovery upon infection with lentiviruses has not been reported. Moreover, these viruses integrate into the host cellular genome and can stay latent without the expression of viral proteins. Latently infected cells serve as a reservoir and fail to be recognized by the immune system, allowing the virus to persist. Thus, the development of effective vaccines against lentiviruses faces additional challenges.

FIV vaccine development

An optimal FIV vaccine should induce long-lasting protective immunity. This immunity should be effective against a wide range of FIV strains within as well as across

subtypes (A-D). In addition, this vaccine should induce protective immunity against cell-free and cell-associated virus and against various routes of infection. Since FIV is predominantly transmitted through biting, protection should be directed in particular against this route of exposure.

In order to properly evaluate the immunogenicity and protective efficacy of FIV vaccines, several factors should be taken into consideration. One factor is the method used to assess the induction of VN antibody responses as these are often used as a parameter for the effectiveness of viral vaccines. The ability of antibodies to neutralize lentiviral infection *in vitro* may however differ with the specificity of the target cell line used in the assay. For example, it has been found that vaccine induced antibodies capable of neutralizing FIV infection on a feline Crandell kidney cell line (CrFK) failed to neutralize FIV infection on feline thymocytes (Siebelink et al. 1993).

Likewise, the assessment of protective efficacy is influenced by several factors. As for viruses in general, these include the route of infection and the dose of challenge inoculum. In most FIV vaccine trials, the challenge inoculum is given either intravenously or intraperitoneally. Experimental infection through these routes is obtained more readily than through mucosal exposure, e.g. requires less viral particles. Furthermore, the origin of the vaccine virus and challenge virus inoculum play an important role in the outcome of vaccine efficacy. Lentiviruses are enveloped and incorporate cellular antigens into the viral envelope when budding from the host cell. These host derived proteins present in the lentiviral envelope may play a role in protective efficacy of inactivated virus and infected cell vaccines, in particular. For example, monkeys immunized with uninfected human cells were shown to be protected from challenge with SIV grown on the identical human cell line. Protected monkeys had significantly higher levels of antibodies directed to host-cell major histocompatibility complex (MHC) antigens than monkeys that were not protected. In fact, protection against SIV has been obtained with vaccines composed of MHC molecules solely (Chan et al. 1992; Stott 1991; Langlois et al. 1992). Likewise, protection

against feline leukemia virus (FeLV), another feline retrovirus, has been afforded in the presence of antibody responses directed against cat-cell antigens (Lee et al. 1982). Thus, the cellular origin of the vaccine virus and the challenge virus should be taken into consideration when evaluating protective efficacy.

In the majority of FIV vaccine trials, the challenge inoculum virus is produced from FIV strains adapted to cell cultures in the laboratory. *In vitro* cell culturing, however, can lead to changes in virulence, cell tropism, and sensitivity to VN antibodies. To overcome this, FIV isolated freshly from infected cats have been used. A relatively new approach is the use of molecularly cloned FIV. The viral genome is incorporated into a plasmid and as such used to infect animals.

FIV vaccine trials

Early FIV vaccine trials utilizing inactivated whole virus failed to demonstrate protective immunity (Table 1.2 and Table 1.3). These trials included the immunization of specific pathogen free (SPF) cats with inactivated FIV UK8 purified from T cells and incorporated into immune stimulating complexes (ISCOMs) (Morein et al. 1984; Hosie 1994). Similarly, an inactivated virus vaccine produced from Crandell feline kidney cells (CrFK) infected with the FIV UT113 isolate and adjuvanted with aluminum hydroxide-oil, failed to protect cats against low dose homologous challenge (Hosie 1994) (Table 1.2).

Initial trials involving inactivated FIV-infected cell vaccines were also unsuccessful. Cats immunized with inactivated FIV UK8 infected T-cells or helper T-cells (Q201) became readily infected upon a homologous challenge with 20 ID_{50} (Hosie 1994, Hosie et al. 1992) (Table 1.3). Comparable results were obtained with a vaccine consisting of inactivated CrFK cells infected with the FIV UT113 isolate (Verschoor et al. 1995). In contrast, partial protection (3 out of 5) was observed in cats immunized with a cell vaccine consisting of FIV_{UT113}-infected thymocytes. However, one of three control cats immunized with

uninfected thymocytes alone also remained virus-negative (Verschoor et al. 1994). Thus, immune responses against cellular antigens and not viral antigens may have been responsible for the observed protection in this study.

Other unsuccessful FIV vaccine trials include those based on FIV subunit proteins and synthetic peptides corresponding to FIV epitopes (Table 1.4). Vaccines composed of nonglycosylated FIV Env produced in *Escherichia coli* and glycosylated Env produced in a baculovirus system failed to protect cats against low dose challenge (Lutz et al. 1995). Similar results were obtained with vaccines consisting of bacterial produced Env fragments fused to galactokinase or glutathione-S-transferase (Verschoor et al. 1996). Also unsuccessful were vaccines composed of synthetic peptides corresponding to the V3 region of the FIV surface envelope protein (SU) (Lombardi et al. 1994). This region resembles the V3 loop of the HIV-1 Env surface protein which is thought to contain the principal neutralizing determinant (PND) of HIV (Pancino et al. 1994). Following immunization, all cats developed V3-specific antibodies however no protection against low dose challenge was observed (Lombardi et al. 1994). Interestingly, immunized animals showed enhancement of infection compared to controls as indicated by a higher virus load in the peripheral blood. Enhancement of infection as a result of immunization was also observed in cats immunized with a FIV envelope produced by recombinant vaccinia virus (Siebelink et al. 1995). In addition, subunit vaccines consisting of recombinant Gag protein p24 or native purified p24, lacked prophylactic efficacy despite the presence of high anti-p24 antibody titers (Hosie et al. 1992).

FIV vaccine trials using recombinant vector based vaccines have also been unsuccessful (Table 1.4). This included a trial in which the efficacy of a replicative defective adenovirus engineered to express the *env* gene of FIV was evaluated. After immunization, Env-specific antibody responses could not be detected and all cats became infected upon challenge (Gonin et al. 1995). Likewise, vaccine protocols involving

Table 1.2 Conventional inactivated whole virus FIV vaccine trials

Type of Immunization	Cellular Origin (Vaccination Route)	Vaccine Virus[a] (FIV subtype)	Vaccine Dose (mg)	Vaccination Protocol(wks)	Type of Adjuvant[b]	Challenge Inoculum		
						Cellular Origin& Route	Dose (ID_{50}) & Strain	Protection rate
(Hosie et al. 1992)								
Whole-virus	feline T-cell(s.c.)	UK-8 (A)	10^{C}	0,5,18	iscom	PBMC (i.p.)	20UK-8	0/5 (0%)
Unvaccinated	(-)	(-)	(-)	(-)	(-)	PBMC (i.p.)	20UK-8	1/4 (25%)
(Verschoor,unpublished)								
Whole virus	CrFK(s.c.)	UT113(A)	100	0,6	AluOH-oil	Thymocytes(s.c.)	10UT113	0/5 (0%)
(Yamamoto et al. 1991h)								
Whole-virus	FL-4 (s.c.)	PET (A)	200	0,2,4,6	CFA/IFA	FeT1 (i.p.)	10PET	3/3(100%)
Whole-virus	FL-4 /Fet[d](s.c.)	PET (A)	$200/10^{7}$	0,2,4,6	CFA/IFA	FeT1 (i.p.)	10PET	2/3 (67%)
Adjuvant alone	(-)	(-)	(-)	0,2,4,6	CFA/IFA	FeT1 (i.p.)	10PET	0/3 (0%)
(Yamamoto et al.1993)								
Whole-virus	FL-4 (s.c.)	PET (A)	250	0,2,5	A-MDP	FeT1 (i.p.)	10PET	13/15 (87%)
Adjuvant alone	(-)	(-)	(-)	0,2,5	A-MDP	FeT1 (i.p.)	10PET	0/10 (0%)
Whole-virus(boost)[e]	FL-4 (s.c.)	PET (A)	250	38e	A-MDP	FeT1 (i.p.)	10DIX(A)	13/13(100%)
Adjuvant alone	(-)	(-)	(-)	38e	A-MDP	FeT1 (i.p.)	10DIX	0/5 (0%)
(Hosie et al. 1995)								
Pelleted-virus	FL-4 (s.c.)	PET (A)	250	0,2,4,7,10,17	T-MDP	FeT1 (i.p.)	10PET	5/6 (83%)
Control	(-)	(-)	(-)	(-)	(-)	FeT1 (i.p.)	10PET	0/6 (0%)
Pelleted-virus	FL-4 (s.c.)	PET (A)	250	0,2,4,7,10,17	T-MDP	Q201(i.p.)	5UK-8	0/5 (0%)
Control	(-)	(-)	(-)	(-)	(-)	Q201(i.p.)	5UK-8	0/5 (0%)
Gradient-purified virus	FL-4 (s.c.)	PET (A)	250	0,3,6	T-MDP	FeT1 (i.p.)	10PET	5/5 (100%)
Control	(-)	(-)	(-)	(-)	(-)	FeT1 (i.p.)	10PET	0/5 (0%)
Gradient-purified virus	FL-4 (s.c.)	PET (A)	250	0,3,6	T-MDP	Q201 (i.p.)	10PET	4/5 (80%)
Adjuvant alone	(-)	(-)	(-)	0,3,6	T-MDP	Q201(i.p.)	10PET	0/5 (0%)
Gradient-purified virus	FL-4 (s.c.)	PET (A)	250	0,3,6	T-MDP	Q201 (i.p.)	10UK-8	1/5 (20%)
Adjuvant alone	(-)	(-)	(-)	(-)	T-MDP	Q201 (i.p.)	10UK-8	0/5 (0%)
(Johnson et al.1995)								
Whole-virus	FL-4 (s.c.)	PET (A)	250	0,2,4,6,8	T-MDP	FeT1 (nasal)	10PET	3/5 (60%)
Adjuvant alone	(-)	(-)	(-)	0,2,4,6,8	T-MDP	FeT1 (nasal)	10PET	0/5 (0%)
Whole-virus	FL-4 (s.c.)	PET (A)	250	0,2,4,6	T-MDP	FeT1 (i.p.)	10SHI (D)	0/2 (0%)
Adjuvant alone	(-)	(-)	(-)	0,2,4,6	T-MDP	FeT1 (i.p.)	10SHI	0/7 (0%)

a UK-8, United Kingdom-8; PET, Petaluma; DIX, Dixon; SHI, Shizuoka.
b isom, immune stimulating complex;; A-MDP, adenyl-muramyldipeptide; CFA, complete Freund's adjuvant; IFA, incomplete Freund's; T-MDP, threonyl-muramyldipeptide.
c Vaccine dose was 10ug of P17 and P24
d Vaccine consisted of 200mg /dose of inactivated whole-virus mixed with $1x10^{7}$ cells/dose of uninfected FeT-1 cells
e Vaccinated cats protected from FIV pet challenge were boosted 38 wks after the first immunization and challenged 3 weeks after the boost with FIVdix strain.

Table 1.3 Conventional inactivated FIV-infected cell vaccine trials

Type of Immunization	Cellular Origin (Vaccination Route)	Vaccine Virus[a] (FIV subtype)	Vaccine Dose (mg/PFU)	Vaccination Protocol(wks)	Type of Adjuvant[b]	Challenge Inoculum		Protection rate
						Cellular Origin& Route	Dose (ID_{50}) & Strain	
(Hosie et al. 1992)								
Infected cell	feline T-cell(s.c.)	UK-8 (A)	$2x10^6$	0,3,6,9,12,15	QuilA	PBMC (i.p.)	20 UK-8	0/5 (0%)
Uninfected cell	(-)(s.c.)	(-)	$2x10^6$	0,3,6,9,12,15	QuilA	PBMC (i.p.)	20 UK-8	1/5 (20%)
(Hosie,unpublished)								
Infected cell	Q201(s.c.)	UK-8 (A)	$1x10^7$	0,3,6	QuilA	Q201 (i.p.)	20 UK-8	0/4 (0%)
Adjuvant alone	(-)(s.c.)	(-)	(-)	0,3,6	QuilA	Q201 (i.p.)	20 UK-8	0/4 (0%)
Control	(-)(s.c.)	(-)	(-)	0,3,6	(-)	Q201 (i.p.)	20 UK-8	1/4 (25%)
(Verschoor et al. 1995)								
Infected cell	CrFK(i.m.)	UT-113 (A)	$2.5x10^7$	0,3,6	alu/MDP	PBMC (i.p.)	10 UT-113	0/5 (0%)
Uninfected cell	CrFK(i.m.)	(-)	$2.5x10^7$	0,3,6	alu/MDP	PBMC (i.p.)	10 UT-113	0/3 (0%)
Infected cell	Thymocytes(i.m.)	UT-113 (A)	$1.5x10^7$	0,3,6	alu/MDP	PBMC (i.p.)	10 UT-113	2/5 (40%)
Uninfected cell	Thymocytes(i.m.)	(-)	$1.5x10^7$	0,3,6	alu/MDP	PBMC (i.p.)	10 UT-113	1/3 (33%)
Control	(-)	(-)	(-)	(-)	(-)	PBMC (i.p.)	10 UT-113	0/2 (0%)
(Yamamoto et al. 1991b)								
Infected cell	Fet1(s.c.)	PET (A)	$1x10^7$	0,2,4,6,8,16	T-MDP	PBMC (i.p.)	10 PET	4/5 (80%)
Infected cell	FL4(s.c.)	PET (A)	$1x10^7$	0,2,4,6,8,16	T-MDP	PBMC (i.p.)	10 PET	2/4 (50%)
Uninfected FeT1	(-)(s.c.)	(-)	$1x10^7$	0,2,4,6,8,16	T-MDP	PBMC (i.p.)	10 PET	0/5 (0%)
Adjuvant alone	(-)(s.c.)	(-)	(-)	0,2,4,6,8,16	T-MDP	PBMC (i.p.)	10 PET	0/5 (0%)
(Yamamoto et al. 1993)								
Infected cell	FL4 (s.c.)	PET (A)	$2.5x10^7$	0,2,5	A-MDP	Fet1 (i.p.)	10 PET	15/15(100%)
Adjuvant alone	(-)(s.c.)	(-)	(-)	0,2,5	A-MDP	Fet1 (i.p.)	10 PET	0/10 (0%)
Uninfected Fet1	FL4 (s.c.)	PET (A)	$2.5x10^7$	38	A-MDP	Fet1 (i.p.)	10 DIX	14/15 (93%)
Adjuvant alone	(-)(s.c.)	(-)	(-)	38	A-MDP	Fet1 (i.p.)	10 DIX	0/5 (0%)
(Matteucci et al. 1995)								
Infected cell	MBM(s.c.)	M-2 (B)	$3x10^7$	0,3,6,9,12,15	IFA	plasma (i.p.)	10 M-2	5/6 (83%)
Uninfected cell	MBM(s.c.)	(-)	$3x10^7$	0,3,6,9,12,15	IFA	plasma (i.p.)	10 M-2	0/3 (0%)
Control	(-)(s.c.)	(-)	(-)	0,3,6,9,12,15	(-)	plasma (i.p.)	10 M-2	0/6 (0%)
(Johnson et al. 1994)								
Infected cell	FI-4(s.c.)	PET (A)	$2.5x10^7$	0,2,4,6,8	A-MDP	Fet1 (nasal)	10 PET	2/5 (40%)
Infected cell	FI-4(s.c.)	PET (A)	$2.5x10^7$	0,2,4,6	A-MDP	Fet1 (i.p.)	20 SHI	1/8 (12%)

a UK-8, United Kingdom 8; UT113, Utrecht 113; PET, Petaluma; M-2, Milan-2; SHI, Shizuoka.

b T-MDP, threonyl-muramyldipeptide; A-MDP, adenyl-muramyldipeptide; IFA, incomplete Freund's adjuvant; QuilA, saponin.

Table 1.4 Subunit and vector-based FIV vaccine trials

Type of Immunization	Cellular Origin (Vaccination Route)	Vaccine Virus[a] (FIV subtype)	Vaccine Dose (mg/PFU)	Vaccination Protocol(wks)	Type of Adjuvant[d]	Challenge Inoculum: Cellular Origin& Route	Challenge Inoculum: Dose (ID_{50}) & Strain	Protection rate
(Hosie, et al. 1992)								
p24	E. coli(s.c.)	UK-8 (A)	50	0,3,5,7	iscom	PBMC (i.p.)	20 UK-8	0/4 (0%)
(Lutz et al. 1995)								
gp100(denatured)	Insect cell (i.m.)[c]	Z2 (A)	100	0,2,4,8	AlOH/QS21	PBMC (i.p.)	20 Z2	0/5 (25%)
gp100(native)	Insect cell (i.m.)	Z2 (A)	100	0,2,4,8	AlOH/QS21	PBMC (i.p.)	20 Z2	1/5 (20%)
gp100(highly purified)	Insect cell (i.m.)	BANG (B)	100	0,2,4,8	AlOH/QS21	PBMC (i.p.)	20 Z2	1/5 (20%)
gp100(native)	Insect cell (i.m.)	BANG (B)	100	0,2,4,8	AlOH/QS21	PBMC (i.p.)	20 Z2	0/5 (0%)
gp100(denatured)	E..Coli (i.m.)	BANG (B)	100	0,2,4,8	AlOH/QS21	PBMC (i.p.)	20 Z2	1/5 (25%)
Control(Ovalbumin)	(-)	(-)	100	0,2,4,8	(-)	PBMC (i.p)	20 Z2	2/7 (29%)
(Osterhaus et al. 1996)								
Env(cleavage)	NA(s.c.)	AM19 (A)	100	0,4,10	iscom	PBMC (i.m)	20AM19	0/6 (0%)
Env(no cleavage)[f]	NA(s.c.)	AM19 (A)	100	0,4,10	iscom	PBMC (i.m.)	20AM19	0/6 (0%)
Env(no cleavage)[f]	NA(s.c.)	AM19 (A)	100	0,4,10	QuilA	PBMC (i.m.)	20AM19	0/6 (0%)
Env-b Gal	NA(s.c.)	AM19 (A)	100	0,4,10	QuilA	PBMC (i.m.)	20AM19	0/6 (0%)
Control(PBS)	(-)(s.c.)	(-)	(-)	0,4,10	(-)	PBMC (i.m.)	20AM19	0/6 (0%)
(Flynn et al. 1995)								
V3-peptide	synthetic (s.c.)	UK-8 (A)	100	0,3,6	QuilA/AlOH	PBMC (i.p.)	10 UK-8	0/15(0%)
Adjuvant alone	(-) (s.c.)	(-)	100	0,3,6	QuilA/AlOH	PBMC (i.p.)	10 UK-8	0/5 (0%)
(Lombardi,1994)								
V3-peptide	synthetic(s.c.)	PET(A)	500	0,2,4,6,8	CFA	FI4(i.v.)	20-PET	0/3 (0%)
Control	(-)	(-)	(-)	(-)	(-)	FI4(i.v.)	20-PET	0/3 (0%)
(Verschoor et al. 1996)								
V3-fusion protein I[e]	E..Coli(s.c.)	UT113 (A)	100	0,4,6,8,10	AlOH	NA(s.c.)	10-20UT113	0/5 (0%)
V3-fusion protein I	E..Coli(s.c.)	UT113	100	0,6,10	QuilA	NA(s.c.)	10-20UT113	0/5 (0%)
Control(PBS)	(-)(s.c.)	(-)	(-)	0,6,10	(-)	NA(s.c.)	10-20UT113	0/5 (0%)
Feline Herpes-Env	(-)oronasal/s.c.	UT113	10^5PFU	0	(-)			
Boost V3-peptide	E. Coli (i.m.)	UT113	100	4,8	AlOH	NA(s.c.)	10-20UT113	0/5 (0%)
Feline Herpes-Env	(-)oronasal/s.c.	UT113	10^5PFU	0	(-)			
Boost V3 peptide	E.Coli (i.m.)	UT113	100	4,8	QuilA	NA(s.c.)	10-20UT113	0/5 (0%)
Feline Herpes-b Gal.	(-)oronasal/s.c.	(-)	10^5PFU	0	(-)			
Boost PBS	(-)(s.c.)	(-)	(-)	4,8	(-)	NA(s.c.)	10-20UT113	0/5 (0%)
(Gonin et al.1995)								
Adenovirus-env	(-)(i.m.)	Wo (A)	11.8-9.2PFU	0,4,30	ISA206	NA(NA)	20 Wo	0/4 (0%)
Adeno-psuedorabies(control)	(-)(i.m.)	(-)	11.8-9.2PFU	0,4,30	ISA708	NA(NA)	20 Wo	0/4 (0%)

a UK-8, United Kingdom; Z2, Zurich; BANG, Bangston; AM19, Amsterdam 19; PET, Petaluma.
b Recombinant vaccinia virus expressed
c Baculovirus expression
d iscom, immune stimulating complex; AlOH/QS21, aluminumhydroxide and non-toxic fraction from Quillaja saponaria; ISA206, water/oil adjuvant;ISA708, water/oil adjuvant.
e V3-fusion protein was composed of the FIV V3 region fused to galactokinase.
f Deletion of the cleavage site between the envelope surface(SU) and transmembrane protein(TM).

priming with a feline herpes virus engineered to express the FIV*env* gene followed by booster immunizations with bacterial Env-fusion proteins failed to induce protection against low dose challenge (Verschoor et al. 1996).

Successful FIV vaccine protocols include the use of inactivated cells infected with the FIV Petaluma isolate (FIV_{Pet}; subtype A) or inactivated cell free FIV_{Pet} (Yamamoto et al. 1991b, 1993). These vaccines were produced from either feline lymphoid cells productively infected with FIV_{Pet} (FL-4) or an IL-2 dependent feline lymphoid cell line (FeT) infected with FIV_{Pet} (Yamamoto et al. 1991a). Using these vaccines, a protection rate of 70%-90% has been observed against low dose experimental challenge with homologous FIV_{Pet} and slightly heterologous FIV Dixon (FIV_{Dix}; subtype A) (less than 9% divergence in the *env* coding region) (Yamamoto et al. 1991b, 1993; Hosie et al. 1995). Further, these vaccines afforded protection against FIV challenge inoculum virus propagated on different cell lines including FeT1, FL-4, and allogeneic PBMC. Protection was achieved against intraperitoneal challenge and oral-nasal challenge in a small number of animals tested (Yamamoto et al. 1991b, 1993; Johnson et al. 1994). These same vaccines, however, failed to induce protection against a high challenge dose of $5x10^4$ ID_{50} with the homologous FIV_{Pet} isolate. Furthermore, these vaccines failed to induce protection against experimental challenge with a moderate heterologous FIV UK8 isolate (subtype-A) and a distinctly heterologous FIV Shizouka (FIV_{Shi}; subtype D) isolate. The Env amino acid sequences of these isolates differ from the FIV_{Pet} Env sequence by 11% and 21%, respectively. In addition, immunization schemes employing a similar vaccine produced from MBM lymphoid cells infected with the Italian isolate FIV M2 have been shown to induce protective immunity against a homologous plasma derived virus inoculum (Mattuecci et al. 1996).

In summary, conventional inactivated vaccines are capable of inducing protective immunity against low dose homologous FIV challenge and slightly heterologous FIV challenge. Similar vaccine approaches have also been successful in other animal models

such as SIV in macaques and HIV in chimpanzees (Desrosiers et al. 1989; Fultz et al. 1992; Murphey-Corb et al. 1989). FIV subunit vaccines consisting of either Env or Gag proteins as well as recombinant vector based vaccines expressing Env did not elicit protective immunity against low dose homologous challenge. In contrast, protective immunity has been obtained against homologous SIV challenge in macaques immunized with Env subunit vaccines and against homologous and heterologous HIV challenge in chimpanzees immunized with Env subunit vaccines (Hu et al. 1992; Girard et al. 1995).

Mechanisms of protection

Protection obtained with conventional inactivated whole virus and infected cell vaccines produced with the FIV_{Pet} isolate, correlated most with high levels of envelope-specific and VN antibodies (Yamamoto et al. 1991b, 1993). Protection obtained with these vaccines did not correlate with anti-MHC antibodies as could be concluded from the lack of protection in cats that were immunized with uninfected cells alone (Yamamoto et al. 1991b, 1993) (Table 1.3). Similar vaccines produced with FIV isolates grown in feline T cells, thymocytes and CrFK cells failed to induce protective immunity (Verschoor et al. 1995; Hosie et al. 1992; Hosie 1994) (Table 1.3). However, these vaccines elicited lower levels of envelope-specific antibodies and VN antibodies. In fact, only the FIV vaccines produced from infected feline T-cell lines FL-4 and FeT1 cells, induced VN antibody titers similar to those observed in infected cats (Tozzinni et al. 1992). This could be due to the larger quantities of envelope protein produced by FL-4 and FeT1 cell lines and the fact that the envelope protein present on FL-4 and FeT1 cells is well preserved following purification. Additional contributing factors may have been the choice of inactivating agent. Inactivation of successful vaccines was accomplished by paraformaldehyde which is thought to be more effective in maintaining antigenicity of immunogens when compared to other inactivating agents i.e. β-propriolactone (Allison and Byars 1991; Warren et al.

1986). Furthermore, the type of adjuvant may have affected the protective efficacy, as it influences the proportion and intensity of humoral vs. cell-mediated responses upon immunization (Byars and Allison 1987).

Additional support for a role of Env-specific and VN antibody responses in the protection obtained with the FIV_{Pet} FL-4/FeT1 vaccines comes from passive immunization studies. In these studies, cats were passively immunized with pooled sera from cats immunized with inactivated FIV_{Pet} infected T-cells (FL-4) or sera from cats experimentally infected with FIV_{Pet} (Hohdatsu et al. 1993). Control cats received either phosphate buffered saline (PBS) or pooled sera from cats immunized with inactivated uninfected FeT1 cells (related to FL-4 cell line) or uninfected 3201 cells (allogeneic feline T-cell line). Upon low dose homologous challenge with 5 ID_{50}, all control cats became infected whereas 3 out of 3 cats passively immunized with FIV_{Pet} (FL-4) vaccine sera and 4 out of 4 cats immunized with FIV infected cat sera did not. Protected cats showed VN antibody titers averaging between 100-200 whereas uninfected cell or PBS control sera had no VN antibody titers. These findings resemble those reported for SIV. Cynomolgus monkeys passively immunized with sera from SIV_{smm} infected and HIV-2 vaccinated monkeys were protected in the presence of high titer antiviral antibodies against homologous challenge at 10 to 100 ID_{50} (Putkonen et al. 1991). In contrast, passive immunization studies in macaques immunized with SIV_{mac} indicated that the levels of anti-cellular and not anti-viral antibodies correlated mostly with protection (Rosenthal et al. 1992).

Findings from studies evaluating protective immunity in kittens born to queens vaccinated with FIV_{Pet} FL-4/FeT1 vaccines also imply a role for VN responses (Pu et al. 1995). In these studies, kittens received colostrum/milk from either vaccinated or sham vaccinated queens and were challenged shortly after birth with low dose homologous challenge of 5 ID_{50}. It was found that only those kittens born to and nursed by vaccinated queens were protected. Furthermore, protected kittens showed high levels of VN titers (500-5000) at five days postparturition. The role of transplancental maternal antibody

reponses was evaluated in kittens born to vaccinated queens and nursed by sham immunized queens. These kittens became infected and showed significantly lower titers of VN (10-100) postparturition as compared to littermate controls receiving colostrum from vaccinated queens. Nevertheless, these kittens showed lower levels of viremia as compared to littermate controls which were born to and nursed by unvaccinated queens (Pu et al. 1995). As such, these data suggest that VN maternal antibodies transferred via colostrum/milk or placenta play a role in preventing establishment of FIV infection. Supporting this are additional studies on kittens born to queens infected for various lengths of time. It was found that kittens born to queens infected for more than 7 months were protected in the presence of high VN titers in the colostrum. In contrast, kittens born to short term (< 2mo.) infected queens became infected in the presence of low VN titers in colostrum (Pu et al. 1995).

Opposing a role for Env specific and VN responses in protection against FIV infection are findings from a number of subunit vaccines trials. Vaccines composed of the FIV envelope or envelope fragments alone failed to induce protective immunity. This included nonglycosylated, glycosylated, native, and denatured whole FIV Env and Env fragment vaccines in combination with different adjuvants (Lutz et al. 1995; Verschoor et al. 1996; Lombardi et al. 1994). The majority of these vaccines, however, effectively induced Env-specific antibody responses as well as VN antibody responses. Therefore, it could be suggested that the tertiary structure of the envelope protein is crucial as it affects the presentation of the Env to the immune system or that other epitopes besides Env are required to obtain protective immunity. A subunit vaccines composed of Gag proteins alone, however, failed to induce protection despite the induction of Gag specific humoral responses (Hosie et al. 1992). Interestingly, several of the envelope subunit vaccines caused enhancement of infection upon challenge (Hosie et al. 1992; Siebelink et al. 1995; Osterhaus et al. 1996). Similar enhancement of viremia has been observed in horses immunized with a recombinant envelope vaccine against equine infectious anemia virus

(EIAV), another lentivirus (Wang et al. 1994). This phenomenon has also been observed with other nonlentiviral vaccines in particular macrophage and monocyte-tropic viruses (Halstead and O'Rourke 1977). It is thought to be mediated by viral specific antibodies elicited upon immunization that can facilitate viral transport to susceptible host cells by binding of the Fc portion of antibodies to the surface of macrophages.

The role of VN antibodies as a factor in preventing FIV infection is not clear. Although, a majority of cats immunized with FIV_{Pet} FL-4/FeT1 vaccines showed high levels of VN antibody responses at challenge, some cats were protected in their absence. In addition vaccine trials employing inactivated MBM cells infected with the FIV M2 isolate, a vaccine similar to the FIV_{Pet} FL-4/FeT1 vaccines, induced protective immunity, in the absence of detectable VN antibody titers (Mattuecci et al. 1996).

In summary, protection against low dose experimental challenge with FIV may in part be mediated by antiviral humoral responses. However, cell-mediated responses such as CTL or underlying immune effector activities i.e. chemokines may also play a role. In fact, recent studies demonstrated the induction of FIV specific CTL responses directed against Env and Gag epitopes in cats immunized with the FIV_{Pet} (Fl-4) inactivated infected cell vaccine (Flynn et al. 1995a). The importance of these responses in protective immunity however remains to be established. Induction of FIV specific CTL responses was also observed in cats immunized with a synthetic peptide vaccine corresponding to the FIV V3 region. These cats, however, became infected upon challenge even though V3-specific CTL responses were detected at the time of challenge (Flynn et al. 1994, 1995b).

Recombinant poxvirus-based vaccines

Members of the poxvirus family comprise a group of large viruses that infect a number of species. Poxviruses are enveloped and contain a single double stranded DNA genome of 130 to 300kbp. These viruses encode their own enzymes required for viral

DNA replication and mRNA synthesis, and are unique in the fact that they replicate within the cytoplasmic compartment of infected cells (Moss 1990).

The use of poxviruses as vaccine vectors was preceded by advances in the field of molecular biology that allowed the manipulation of viruses in such a way that foreign genes could be inserted and expressed (Piccini et al. 1987; Perkus et al. 1989). Poxviruses have since become candidate vaccine-vectors for a wide variety of pathogens (Perkus et al. 1995). In comparison to other candidate vector-viruses, poxviruses are exceptionally well-suited due to their physical stability, low production costs, and ease of administration. Furthermore, poxviruses have large genomes that allow the insertion of multiple genes.

The most widely used member of the poxvirus family is the vaccinia virus, prototype of the Orthopoxviruses (Esposito 1991). This virus has been engineered to express antigens of bacterial and viral pathogens and shown to induce protective immunity *in vivo* (Perkus et al. 1995). Immune-responses induced upon inoculation with vaccinia based vaccines include both humoral responses and cell mediated responses directed against the inserted foreign antigens. There are however some concerns about the safety of vaccinia when used in a large population. As vaccinia exhibits a broad host-range there is a potential risk of spread to the general environment. Moreover, vaccinia has been shown to cause disseminated infection in immuno-compromised people (Fulgitini et al. 1968).

For these reasons, the development of poxviruses as vector vaccines has been extended to attenuated poxviruses and poxviruses with a more restricted host-range. One such example is the NYVAC vector. This vector was derived from the Copenhagen vaccinia strain by the selective deletion of 18 open reading frames, encoding genes involved in host-specificity and virulence (Tartaglia et al. 1992). These deletions resulted in a virus which replication is highly impaired on cell lines from several species including human cells. Furthermore, this virus lacks virulence in immuno-compromised animal models. The MVA vaccinia strain is another example of an attenuated vector strain used in vaccine development. This strain was derived by extensive passaging of the Ankara

vaccinia strain on primary chick embryo-fibroblast. As a result, the MVA virus is severely attenuated and lacks replication on nonavian cell lines (Sutter et al. 1994).

An alternative to the use of attenuated vaccinia viruses is the use of poxviruses that exhibit species specificity (Baxby and Paoletti 1992). These include the suipoxviruses, capripoxviruses and avipoxviruses. Avipoxviruses only productively infect cells of avian origin and were originally developed as vaccine vectors for the poultry industry. Unexpectedly, it was found that nonavian cells inoculated with avipoxvectors expressed the inserted antigen despite the absence of vector replication (Taylor et al 1992b). Moreover, it was found that these vectors when administered to nonavian species were capable of eliciting protective immunity. It is now understood that these viruses undergo abortive infection in nonavian cells resulting in expression of early gene and inserted gene products.

Two avipoxviruses, the fowlpoxvirus and canarypoxvirus, have been developed as vaccine vectors (Plotkin et al. 1995). ALVAC represents a vector derived from an attenuated canarypoxvirus strain originally used to immunize canaries. The protective potential of recombinant ALVAC vectors has been tested against several viral pathogens including rabies, measles, Japanese encephalitis virus (JEV), cytomegalovirus (CMV), and equine influenza virus (EIV) (Table 1.5) (Cadoz et al. 1992; Konishi et al. 1994; Taylor et al. 1992a, 1992b; Gonczol et al. 1995). The majority of these ALVAC-based vaccines were shown to elicit antigen-specific antibody responses, including VN antibody responses specific for the inserted antigens. More important, cell-mediated immunity was also induced in individuals immunized with these vaccines. In one study, lymphocytes from human volunteers immunized with an ALVAC-rabies recombinant had proliferative responses to the rabies antigens, demonstrating the induction of antigen specific T-helper cells (Cadoz et al. 1992; Taylor et al. 1991). This same recombinant vaccine was used to evaluate the ability of ALVAC-based vaccines to elicit memory immune responses in dogs.

Table 1.5 Immunogenicity and prophylactic efficacy of ALVAC-based vaccines

Pathogen	genus	Test species	Humoral responses	Cell-mediated responses	Protection
Rabies[a]	Rhabdoviridae	mice	+	ND	+
		dog	+	ND	+
		cats	+	ND	+
		squirrel monkeys	+	ND	ND
		rhesus macaques	+	ND	ND
		chimpanzees	+	ND	ND
		humans	+	ND	ND
Cytomegalovirus[b]	Herpesviridae	mice	+	+	ND
		guinea pigs	+	+	ND
Canine distemper virus[c]	Paramyxoviridae	dogs	+	ND	+
Japanese encephalitis virus[d]	Flaviviridae	mice	+	ND	+

[a] Cadoz et al. 1992; Taylor et al. 1991.
[b] Gonczol et al. 1995.
[c] Taylor et al. 1992a, 1992b.
[d] Konishi et al. 1994.
ND=not determined

It was found that dogs could be protected from a rabies challenge given 36 weeks after a single ALVAC-rabies immunization (Cadoz et al. 1992).

The efficacy of ALVAC-based vaccines has also been tested against a number of retroviral pathogens (Table 1.6). Immunization with ALVAC recombinants expressing the Envelope and Gag proteins of the feline leukemia virus (FeLV) protected cats against experimental infection with FeLV (Tartaglia et al. 1993). Protection was afforded in the absence of detectable VN antibody responses. Interestingly, protected animals developed FeLV-specific VN antibody titers at 9-12 weeks postchallenge whereas control animals failed to develop VN antibody titers. The data presented in this study should be interpreted with some caution since no analysis of FeLV by PCR was performed on tissues of the protected animals (Tartaglia et al. 1993). Thus, immunization may not have resulted in sterilizing immunity but resulted in a reduced viral load explaining the development of VN titers at 9-12 weeks postchallenge. Further, the evaluation of cell-mediated responses was not included in this study.

ALVAC-based vaccines have protective efficacy against human T cell leukemia/lymphoma virus type I (HTLV-1), the causative agent of adult T cell leukemia and tropical spastic paraparesis in humans (Barre-Sinoussi et al. 1983). In the rabbit model used, it was found that two inoculations with ALVAC-based vaccines encoding the HTLV-1 envelope protein (ALVAC-gp65), protected rabbits against challenge infection with HTLV-1 infected cells. Protective immunity was afforded in the absence of HTLV-1 VN antibody responses. Again, this study lacked the evaluation of cell-mediated reponses. Interestingly, rabbits boosted with baculovirus expressed envelope protein (gp65) in addition to ALVAC-gp65 immunizations failed to be protected (Franchini et al. 1995b).

The rhesus macaque model was used to assess the prophylactic efficacy of ALVAC-based vaccines against HIV-2 challenge (Franchini et al. 1995a). It should be kept in mind that rhesus macaques can be infected with HIV-2 but do not develop an AIDS like syndrome. Rhesus macaques were given two immunizations with ALVAC

recombinants expressing HIV-2 Env and Gag/Prot followed by two immunizations with recombinant Env (gp160) and a final ALVAC-HIV-2 Env, Gag/Prot boost. Upon challenge with 100 ID_{50} HIV-2, both macaques were free of viremia and tested negative for the virus by virus isolation and virus specific PCR. Prior to challenge, HIV-2-specific CTL responses were detected. In addition, one of the macaques had a transient VN antibody titer prior to challenge. After challenge both animals developed significant VN antibody titers as such resembling findings in the ALVAC-FeLV trials (Franchini et al. 1995a; Tartaglia et al. 1993). The macaques were given a second HIV-2 challenge of 100 ID_{50} at 7 months post the primary challenge without any intervening boosters. At this time, both macaques became infected despite the presence of VN antibody titers at the time of challenge. Cytotoxic T-lymphocyte responses were not measured at the time of the second challenge, however, both animals were positive for HIV-2 specific CTL activity at one month following the second challenge.

To evaluate the strain specificity of the immunity generated upon immunization with ALVAC-based vaccines, rhesus macaques were immunized with an ALVAC-recombinant expressing Env, Gag/Prot of the HIV-1_{MN} isolate and challenged with a distinctly heterologous HIV-2 isolate (> 40% difference) (Abimiku et al. 1995). Two rhesus macaques received a combination of primary ALVAC-HIV-1_{MN} immunization and subsequent subunit boosts with either p24 and gp160 or a tandem V3-peptide. Both animals developed virus-specific CTL and VN antibodies after immunization. Upon challenge, the animal boosted with the V3-peptide was considered partially protected, based on the low VN titers and the absence of virus by virus isolation and PCR at six months postchallenge. The other animal exhibited a delay by two months and had lower VN antibody titers as compared to control animals. All control animals became positive at 1 month after challenge and remained virus positive throughout the study. Perhaps the most interesting observation made in this study was that the VN antibody responses generated upon immunization did not cross react with the HIV-2 isolate used in the challenge

inoculum. Based on this it was speculated that cell mediated responses attributed to the partial protection observed in these animals. However, the assessment of cross-reactive CTL responses to the HIV-2 isolate was not included in this study.

In addition to the macaque model, the chimpanzee model has been used to assess the prophylactic efficacy of ALVAC-based vaccine protocols against HIV infection. In one study, the efficacy of an ALVAC recombinant expressing both the HIV-1_{LAI} Env and Gag (ALVAC-HIV_{LAI} gp120TM, Gag/Prot) was evaluated against cell-associated HIV-$1_{IIIB(LAI)}$ challenge in two chimpanzees. Each animals received a total of five immunizations and were challenged one month after the final immunization. HIV-specific antibody responses including VN antibodies were detected after the 4th and 5th immunization in both animals. Upon challenge, one animal remained virus negative and the other, including a naive control animal, became infected. Interestingly, the protected animal had a higher VN antibody titer at the time of challenge than the nonprotected animal (Van der Ryst et al. 1996). Importantly, these same vaccine protocols failed to induce protective immunity against challenge with the heterologous HIV_{DH12} isolate (personal communication).

Further, the vaccine efficacy of ALVAC-HIV_{LAI} gp120TM, Gag/Prot recombinants was evaluated against mucosal challenge in chimpanzees. One group consisting of two animals was immunized by the intra-muscular (i.m.), cervico-vaginal, and rectal route simultaneously. Another group was immunized by the i.m., oral and nasal route and one animal was immunized by the i.m. route. All animals were challenged intra-cervically with 2500 $TCID_{50}$ of HIV-1_{LAI} passaged in chimpanzees. All vaccinated animals were free from virus infection whereas the nonvaccinated control animals were infected. HIV-specific antibody responses, including VN antibody titers, were low or undetectable at the time of challenge, thus implying that mucosal protection occurred through mechanisms other than VN antibody responses (Girard et al. 1996).

Another study reported on the efficacy of ALVAC-based vaccine strategies against heterologous HIV challenge in chimpanzees. Chimpanzees were immunized with an

ALVAC recombinant vaccine encoding Env of the HIV-1_{MN} strain and boosted with recombinant envelope glycoproteins (gp160) of the HIV-1_{MN} and HIV-1_{LAI}, both classified as subtype B strains. The animals were challenge intravenously with HIV-1_{SF2} which is also classified as a subtype B virus but differs significantly from the HIV-1_{LAI} strains. Upon challenge, virus was isolated from both vaccinated animals. However, in comparison to the infected control animals the vaccinated animals had a lower viral load (Girard et al. 1995)

In summary, ALVAC-based recombinants were effective against lentiviral infection in several animal models. However, it is not clear as to what constituted vaccine protection. Some of these studies suggest a role for VN antibody responses. On the other hand, other studies showed protection in the absence of detectable VN antibody responses. Conflicting results may also stem from the fact that only a limited number of animals was used in these trials. Furthermore, the assessment of immune responses, in particular cell-mediated responses, was frequently omitted in these trials. Nevertheless, the data obtained from these animal trials are promising and studies on the safety and immunogenicity of these vectors in human volunteers have been initiated. Thus far, no adverse effects have been reported in human subjects immunized with an ALVAC-recombinant expressing HIV Env (gp160), ALVAC-HIV$_{MN}$gp160, followed by a boost with recombinant gp160 (Clements et al. 1996, Lawrence et al. 1996). Further, it was found that immunization with this ALVAC recombinant alone failed to induce VN antibody responses. However, VN antibodies were detected in most subjects after the rgp$160_{MN/LAI}$ boost. Env-specific T-cell proliferative responses were detected in a small percentage of subjects after ALVAC immunizations and in all subjects following rgp$160_{MN/LAI}$ boosts. The presence of HIV Env-specific CTL activity was detected in some of the subjects, even without the subunit boost (Pialoux et al. 1995). In a similar study, immune responses elicited with ALVAC-HIV$_{MN}$gp160 alone was compared to those elicited by ALVAC-HIV$_{MN}$gp160 priming followed by a HIV-1 rgp120_{SF2} boost. Boosting with envelope protein significantly

enhanced VN antibody responses, ADCC, and CTL responses as compared to immunization with either ALVAC-HIV_{MN}gp160 or $rgp120_{SF2}$ alone (Clements et al. 1996).

In a follow-up study, volunteers received immunizations with an ALVAC recombinant expressing both HIV-1 Env and Gag and a boost with recombinant Env (Pialoux et al. 1995; Clements et al. 1996). The immunogenicity resembled that of the previous studies (Lawrence et al. 1996). Interestingly, Gag-specific CTL responses were detected in the majority of subjects whereas Env-specific CTL-responses were only detected in a small percentage of the subjects. Together, these studies demonstrate that immunization schemes involving ALVAC-based HIV vaccines, in combination with whole protein boosts, are safe and can elicit both humoral and cell-mediated responses specific for the inserted immunogens.

Table 1.4 Subunit and vector-based FIV vaccine trials

Type of Immunization	Cellular Origin (Vaccination Route)	Vaccine Virus[a] (FIV subtype)	Vaccine Dose (mg/PFU)	Vaccination Protocol(wks)	Type of Adjuvant[d]	Challenge Inoculum		Protection rate
						Cellular Origin& Route	Dose (ID_{50}) & Strain	
(Hosie, et al. 1992)								
p24	E. coli(s.c.)	UK-8 (A)	50	0,3,5,7	iscom	PBMC (i.p.)	20 UK-8	0/4 (0%)
(Lutz et al. 1995)								
gp100(denatured)	Insect cell (i.m.)[c]	Z2 (A)	100	0,2,4,8	AlOH/QS21	PBMC (i.p.)	20 Z2	0/5 (25%)
gp100(native)	Insect cell (i.m.)	Z2 (A)	100	0,2,4,8	AlOH/QS21	PBMC (i.p.)	20 Z2	1/5 (20%)
gp100(highly purified)	Insect cell (i.m.)	BANG (B)	100	0,2,4,8	AlOH/QS21	PBMC (i.p.)	20 Z2	1/5 (20%)
gp100(native)	Insect cell (i.m.)	BANG (B)	100	0,2,4,8	AlOH/QS21	PBMC (i.p.)	20 Z2	0/5 (0%)
gp100(denatured)	E..Coli (i.m.)	BANG (B)	100	0,2,4,8	AlOH/QS21	PBMC (i.p.)	20 Z2	1/5 (25%)
Control(Ovalbumin)	(-)	(-)	100	0,2,4,8	(-)	PBMC (i.p)	20 Z2	2/7 (29%)
(Osterhaus et al. 1996)								
Env(cleavage)	NA(s.c.)	AM19 (A)	100	0,4,10	iscom	PBMC (i.m)	20AM19	0/6 (0%)
Env(no cleavage)[e]	NA(s.c.)	AM19 (A)	100	0,4,10	iscom	PBMC (i.m.)	20AM19	0/6 (0%)
Env(no cleavage)[f]	NA(s.c.)	AM19 (A)	100	0,4,10	QuilA	PBMC (i.m.)	20AM19	0/6 (0%)
Env-b Gal	NA(s.c.)	AM19 (A)	100	0,4,10	QuilA	PBMC (i.m.)	20AM19	0/6 (0%)
Control(PBS)	(-)(s.c.)	(-)	(-)	0,4,10	(-)	PBMC (i.m.)	20AM19	0/6 (0%)
(Flynn et al. 1995)								
V3-peptide	synthetic (s.c.)	UK-8 (A)	100	0,3,6	QuilA/AlOH	PBMC (i.p.)	10 UK-8	0/15(0%)
Adjuvant alone	(-) (s.c.)	(-)	100	0,3,6	QuilA/AlOH	PBMC (i.p.)	10 UK-8	0/5 (0%)
(Lombardi,1994)								
V3-peptide	synthetic(s.c.)	PET(A)	500	0,2,4,6,8	CFA	Fl4(i.v.)	20-PET	0/3 (0%)
Control	(-)	(-)	(-)	(-)	(-)	Fl4(i.v.)	20-PET	0/3 (0%)
(Verschoor et al. 1996)								
V3-fusion protein I[e]	E..Coli(s.c.)	UT113 (A)	100	0,4,6,8,10	AlOH	NA(s.c.)	10-20UT113	0/5 (0%)
V3-fusion protein I	E..Coli(s.c.)	UT113	100	0,6,10	QuilA	NA(s.c.)	10-20UT113	0/5 (0%)
Control(PBS)	(-)(s.c.)	(-)	(-)	0,6,10	(-)	NA(s.c.)	10-20UT113	0/5 (0%)
Feline Herpes-Env	(-)oronasal/s.c.	UT113	10^5PFU	0	(-)			
Boost V3-peptide	E. Coli (i.m.)	UT113	100	4,8	AlOH	NA(s.c.)	10-20UT113	0/5 (0%)
Feline Herpes-Env	(-)oronasal/s.c.	UT113	10^5PFU	0	(-)			
Boost V3 peptide	E.Coli (i.m.)	UT113	100	4,8	QuilA	NA(s.c.)	10-20UT113	0/5 (0%)
Feline Herpes-b Gal.	(-)oronasal/s.c.	(-)	10^5PFU	0	(-)			
Boost PBS	(-)(s.c.)	(-)	(-)	4,8	(-)	NA(s.c.)	10-20UT113	0/5 (0%)
(Gonin et al.1995)								
Adenovirus-env	(-)(i.m.)	Wo (A)	11.8-9.2PFU	0,4,30	ISA206	NA(NA)	20 Wo	0/4 (0%)
Adeno-psuedorabies(control)	(-)(i.m.)	(-)	11.8-9.2PFU	0,4,30	ISA708	NA(NA)	20 Wo	0/4 (0%)

a UK-8, United Kingdom; Z2, Zurich; BANG, Bangston; AM19, Amsterdam 19; PET, Petaluma.
b Recombinant vaccinia virus expressed
c Baculovirus expression
d iscom, immune stimulating complex; AlOH/QS21, aluminumhydroxide and non-toxic fraction from Quillaja saponaria; ISA206, water/oil adjuvant;ISA708, water/oil adjuvant.
e V3-fusion protein was composed of the FIV V3 region fused to galactokinase.
f Deletion of the cleavage site between the envelope surface(SU) and transmembrane protein(TM).

CHAPTER II
IMMUNOGENICITY AND PROTECTIVE EFFICACY EVALUATION OF CANARYPOXVIRUS (ALVAC)-BASED FIV VACCINES AGAINST HOMOLOGOUS FIV CHALLENGE

Introduction

The feline immunodeficiency virus (FIV) is the causative agent of an immunodeficiency syndrome in cats (Pedersen et al. 1987). The immunological and pathological changes observed in FIV infected cats closely resemble those observed in humans infected with HIV the causative agent of AIDS (Yamamoto et al. 1988b; Ackley et al. 1990). Based on these similarities FIV infection in cats has become a valuable model for the evaluation of vaccine and prophylactic strategies. Similar to HIV, it is still unknown what constitutes protective immunity against FIV. However, vaccine protection against experimental FIV infection in cats has been achieved with conventional vaccines such as inactivated whole-virus and inactivated FIV-infected cell vaccines (Yamamoto et al. 1991, 1993; Hosie et al. 1995; Verschoor et al. 1995). This vaccine approach has also been successful against lentiviral infection in other animal models, such as SIV in macaques and HIV in chimpanzees (Murphey-Corb et al. 1986; Fultz et al. 1992). The use of such vaccine approach in humans, however, may not be feasible because of safety issues, such as incomplete inactivation of vaccine virus which could potentially lead to infection of immunized subjects.

An alternative to the use of conventional vaccines, is the use of viral vectors based vaccines which can be engineered to encode specific components of these viruses. One

vector evaluated in current trials of HIV vaccines, is the canarypoxvirus vector, ALVAC (Baxby et al. 1992; Perkus et al. 1995).Canarypoxvirus (ALVAC)-based vaccines are considered safe due to their restricted host-range and their inability to undergo full-replication in cells of non-avian origin (Baxby et al. 1992). The efficacy of ALVAC-based vaccines against lentiviruses has been tested in several animal models. Rhesus macaques immunized with ALVAC-recombinants expressing Env and Gag of HIV-2 were protected from infection with homologous HIV-2 (Franchini et al. 1995a). Additionally, ALVAC-HIV-1 vaccines have been proven effective against experimental infection with homologous HIV-1 in a small number of chimpanzees tested (Girard et al. 1995, 1996; Van der Ryst et al. 1996). Although, the number of animals in these trials was small, these findings suggest that ALVAC-based vaccines can induce protective immunity against lentiviral infection.

Hence, the main objective of this study was to assess the immunogenicity and protective efficacy of recombinant canarypoxvirus (ALVAC)-vectored FIV vaccines alone or in combination with conventional inactivated FIV-infected cell vaccines. Four distinct ALVAC-FIV recombinants were tested. This included recombinants that encoded the FIV Env (ALVAC-*env*), the FIV Gag and Prot (ALVAC-*gag/prot*) or both the FIV Env, Gag and Prot (ALVAC-*env,gag/prot*). Also included was a recombinant that encoded the FIV Gag and a modified FIV Env from which a putative immunosuppressive region had been deleted (ALVAC-*97TMG*).

This study was conducted to address the following specific aims:

I. *In vitro* evaluation of ALVAC-FIV recombinant expression.

Determine if ALVAC-FIV recombinants are able to infect non-avian, non-permissive, feline cells and properly express the inserted FIV gene constructs in these cells.

II. *In vivo* evaluation of ALVAC-FIV recombinant vaccines.

a) Identify the immune responses, both humoral and cell-mediated, elicited in cats after immunization with ALVAC-FIV recombinants alone or after priming with ALVAC-FIV recombinants followed by boosting with inactivated FIV-infected cell vaccine (ICV).

b) Assess the protective efficacy of immunization protocols employing ALVAC-*env*, ALVAC-*gag/prot*, ALVAC-*97TMG* and ALVAC-*env,gag/prot* against experimental infection with the homologous subtype FIV Petaluma (FIV_{Pet}) isolate in cats.

c) Assess the efficacy of a combination prime-boost protocol consisting of ALVAC-*env,gag/prot* priming followed by inactivated FIV-infected cell boost, against experimental infection with the homologous subtype FIV_{Pet} isolate in cats.

Materials and Methods

Construction of ALVAC-recombinants

ALVAC is a canarypoxvirus vector derived from a canarypoxvirus vaccine strain used to immunize canaries, Kanapox (Rhone-Merieux, Lyon, France). The ALVAC-FIV recombinants were generated using standard procedures similar to those used by Virogenetics to generate ALVAC-based FeLV recombinants (Piccini et al. 1987; Tartaglia et al. 1993). Briefly, the coding region of the $FIV_{Ville\ franche}$ isolate (subtype A) *env*, *gag* and *prot* were amplified by polymerase chain reaction (PCR) and fused in a precise ATG to ATG fashion with vaccinia early/late promotors H6 or I3L, respectively (Figure 2.1). The protease gene was included in the constructs to ensure proper proteolytic cleavage of the Gag precursor protein into the mature matrix protein (MA, p15), capsid protein (CA, p24)

and nucleocapsid protein (NC, p7). The promotor-FIV gene constructs were then cloned into the pC6L donor plasmid that contained ALVAC C6 flanking regions to enable insertion of the FIV promotor-gene constructs into the ALVAC non-essential C6 genetic locus. Prior to insertion into the donor plasmid, TsNT sequence elements were removed from the FIV coding sequences without altering the amino acid sequence. TsNT motifs are recognized by the poxvirus transcriptional apparatus as termination signals at early times postinfection and their retention is known to result in diminished expression of foreign gene products (Yuen and Moss 1987; Earl et al. 1990). The donor plasmid was then transfected into ALVAC-infected chicken embryo fibroblast cells to generate full ALVAC-recombinants by homologous recombination. Recombinants were subjected to several rounds of plaque purification and analyzed by nucleotide sequence analysis to ensure proper insertion.

ALVAC-FIV recombinants generated for this trial included ALVAC-*env* containing the whole FIV *env* coding region, ALVAC-*gag/prot* containing the FIV *gag* and *prot* coding regions and ALVAC-*env,gag/prot* containing the FIV *env*, *gag* and *prot* coding regions. The ALVAC-*97TMG* recombinant contained the FIV *gag* and *prot* gene and a modified *env* coding region, of which a 714bp fragment encoding a putative immunosupressive element, had been deleted (Figure 2.1).

In vitro Expression of the FIV Env and Gag

The ability of ALVAC-FIV recombinants to infect non-permissive non-avian cells was evaluated on CrFK cells, a feline fibroblastic kidney cell line. Petri dishes seeded with a monolayer of CrFK cells were inoculated with ALVAC-FIV recombinants or ALVAC vector alone at a multiplicity of infection (m.o.i.) of 10 for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. The ALVAC inoculum was removed and cells were incubated with fresh medium for an additional 48 h at 37°C. At 48 h, cells were removed

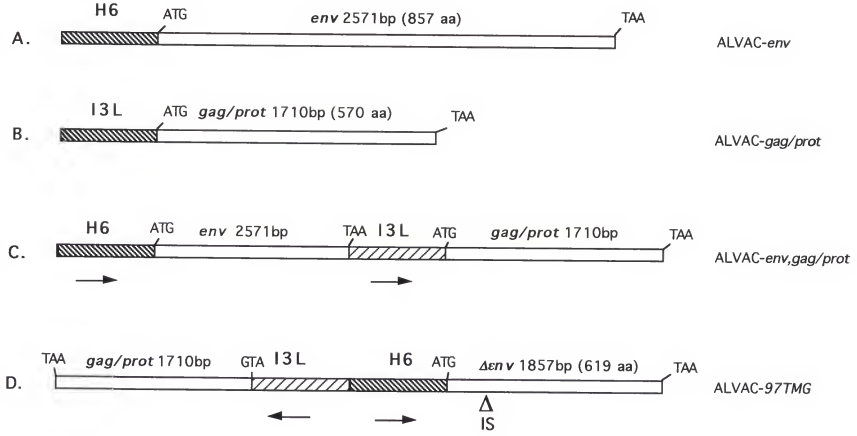


Figure 2.1 Schematic representation of ALVAC constructs

by scraping and washed three times in phosphate-buffered saline (PBS). DNA was extracted from the cells by resuspending in lysis buffer consisting of 0.021 M Tris (pH 7.5), 0.029 M EDTA (pH 8.0), 0.1 M NaCl, 1% SDS and proteinase K (10 mg/ml). Cells in lysis buffer were incubated at 56°C for 3-4 h followed by a 20 min incubation at 95°C to inactivate Proteinase K activity. The presence of FIV-specific DNA encoded by the ALVAC-recombinants in the obtained DNA samples was determined by PCR using FIV *env*- and *gag*-specific primers [FIV *env* specific primers 5'-GAAATGTATAATATTGCTGG-'3 and 5'-GAATTGATTTTGATTACATCC-'3; 5'-GGTAGGAGAGATTCTACA-'3 and 5'-CTGCATTCTTGCTGGTGC-'3 FIV *gag* specific primers]. PCR reactions were carried out in a final volume of 50 µl containing: 1.0 µg DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dTTP and dGTP), 20 pmol of each primer, and 2.5 units of *Taq* DNA polymerase. The reaction mixtures were incubated for 5 min at 94°C and cycled 30 times through 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C, followed by 10 min incubation at 72°C. Finally, the PCR reaction products were separated by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide.

Detection of FIV-specific mRNA transcripts by RT-PCR

The expression of messenger RNA corresponding to the FIV genes encoded within the ALVAC-recombinants was evaluated in non-permissive CrFK cells. Monolayers of CrFK cells were inoculated with ALVAC-FIV recombinants or ALVAC vector alone as described above. At 48 h postinoculation, messenger RNA was isolated using the Micro-Fast track mRNA isolation kit (Invitrogen, San Diego, CA). The isolated mRNA was then reverse transcribed into cDNA. Briefly, 1 µg of isolated mRNA was mixed with 7 µl DEPC-treated H_20 and 2 µl pdN_6 and incubated at 65°C for 5 min. Subsequently, the samples were mixed with 4 µl 5X buffer, 1.3 µl DTT, 0.7 µl RNase inhibitor (40 U/µl), 1 µl dNTPs (10 mM dATP, dGTP, dTTP, dGTP), 2.0 µl acetylated BSA and 2 µl M-MLV

reverse transcriptase (Superscript RT 200 U/μl, Rnase H, Gibco BRL, Gaithersburg, MD) and incubated at 42°C for 1 h, followed by 10 min at 95°C. The generated cDNA served as a template for PCR reactions using *env* and *gag* specific primers. PCR was performed as described above.

Indirect immunofluorescence

Protein expression of the ALVAC encoded FIV *env* and *gag* genes was analyzed by indirect immunofluorescence on CrFK cells inoculated with ALVAC-FIV recombinants. CrFK were seeded at a density of $5x10^5$ cells per $35mm^2$ dish on sterile glass coverslips and infected at a m.o.i. of 10 with ALVAC-FIV recombinants or the ALVAC vector alone. Indirect immunofluorescence was performed at 48 h postinoculation. Cells were fixed in 4% paraformaldehyde for 10 min, washed in PBS, and permeabilized in PBS containing 0.2% Triton X-100. Cells were then incubated with pooled FIV-positive serum for 30 min, washed, and incubated with FITC-labeled anti-cat IgG. All serum and antibody dilutions were made in PBS containing 3% bovine serum albumin (BSA). Finally, cells were washed and counterstained with Evans Blue (0.5% in PBS) for 10 min, observed under a microscope and photographed.

ALVAC vaccine production and titering

Both ALVAC vector and ALVAC-FIV recombinants were amplified on permissive primary chicken embryo fibroblast (CEF) and titered *in vitro* by measuring the number of plaque forming units (PFU). Vaccines were produced from clarified lysates of infected CEF cells in serum free medium and aliquoted at $1X10^8$ PFU per dose.

Inactivated-cell vaccine preparation and titering

The inactivated FIV-infected cell vaccine (ICV) was produced from an IL-2 independent feline lymphoid cell line (FL-4) chronically infected with the FIV Petaluma isolate (subtype A). This cell line was cloned from an IL-2 dependent feline T-cell line (FeT1) infected with FIV_{Pet} and stains positive for CD8,CD4 and PanT surface markers and negative for IgM heavy and light chains (Yamamoto et al. 1991a). The ICV vaccine was generated by inactivation of FL-4 cells with 1.25% paraformaldehyde for 24 h, followed by extensive dialysis against PBS. A single vaccine dose consisted of $2.5x10^7$ fixed infected cells mixed with 250μg SAF/muramyl dipeptide (MDP) (Chiron Corporation).

Animals

A total of 36 specific pathogen free (SPF) cats (*Felis catus*, domestic short hair), 12 weeks of age, were purchased from Liberty Research Inc. (Waverly, NY). The animals were housed at the Infectious Disease complex of Animal Resource Services and cared for in accordance with the policies set by the Environmental Health and Safety division (EH&S) and the Animal Care Committee of the University of Florida. All cats received a combination vaccine against feline herpes virus, calicivirus and panleukopeniavirus (Fel-O-Vax, Ford Dodge laboratories, Mason City, IA). Animals were not vaccinated against feline leukemia virus (FeLV). Prior to immunization, all animals tested negative for *Toxoplasma gondii* , FeLV and FIV by immunoblot analysis.

Grouping and Immunization Protocol

Cats were divided into 7 groups, with equal numbers of males and females in each group (Table 2.1). Littermates were evenly spread over all groups. Cats were immunized

Table 2.1 Grouping and Immunization

Group	Cat ID#	sex	Vaccine(Number of Immunizations)
	QH4	F	
	PY1	M	
Group A	QO1	F	ALVAC-*env* (3X)
	QC1	M	
	QU1	M	
	QL2	F	
	QQ1	M	
	QA5	F	
Group B	QU2	F	ALVAC-*gag/prot* (3X)
	QX3	M	
	QI1	M	
	QL3	F	
	QH5	F	
	PY3	M	
Group C	QS4	F	ALVAC-*env,gag/prot* (3X)
	QC3	M	
	QG3	F	
	QE2	M	
	QQ2	M	
	PY5	F	
Group D	QO2	F	ALVAC-*97TMG* (3X)
	QX4	M	
	QI2	M	
	QL4	F	
	QH2	M	
	PY2	M	
Group E	QA4	F	ALVAC (3X)
	QC4	M	
	QG5	F	
	QE3	F	
	QH3	M	
Group F	PY4	M	ALVAC-*env,gag/prot* (2X)
	QA6	F	ICV (1X)
	QC5	F	
Group G	QG4	F	ALVAC (2X)
	QE4	M	& ICV (1X)

F=Female M=Male

a total of three times at monthly intervals. The ALVAC vaccine was administered intramuscularly at $1x10^8$ PFU/cat. The inactivated FIV-infected cell vaccine (ICV) was mixed with 250 μg SAF/MDP adjuvant and was administered subcutaneously.

Challenge

The challenge inoculum consisted of cell-free culture fluid from PBMC infected with FIV_{Pet}, previously titered *in vivo* in SPF cats. The challenge inoculum of 50 ID_{50} was given intraperitoneally (i.p.) four weeks after the final immunization.

FIV immunoblot assay

Sucrose gradient purified FIV_{Pet} from chronically infected FL-4 lymphoid cells was separated by a 10% SDS-polyacrylamide gel (SDS-PAGE). Proteins were transferred to nitrocellulose sheets (pore diameter of 0.45 μm) by wet blotting. After transfer, the sheets were blocked for 1-2 h at 37°C in gelatin buffer (PBS containing 3% gelatin and 0.02% sodium azide) and cut into strips. Serum samples of immunized and non-immunized cats were diluted at 1:100 in Buffer 3 (0.05 Tris at pH7.4 containing 0.15 M sodium chloride, 0.001 M EDTA, 0.05 % Tween-20, and 1 % BSA) and incubated with immunoblot strips for 4 h at 37°C. The reactions were stopped with $ddiH_2O$ and strips were washed 3 times with ELISA buffer (see ELISA protocol). The strips were incubated with biotinylated anti-cat IgG (Southern Biotechnology) for 1 h at 37°C followed by three washes with ELISA buffer. Strips were then incubated with streptavidin conjugated to horseradish peroxidase for 1 h at 37°C. The reactions were stopped and washed 3 times with ELISA buffer. Finally, strips were incubated with fresh substrate solution (0.1 M Tris at pH7.4 containing 0.05% diamino benzidine, 400 mg/ml of $NiCl_2$ and 0.01% H_2O_2). Upon appearance of visible bands, reactions were stopped with an excess of $ddiH_2O$.

The titer of FIV-specific antibodies, if detected, was determined by testing 10-fold serial dilutions of serum samples (1:100 to 1:1000000) as described above and defined as the reciprocal of the highest dilution (in ^{10}Log) at which FIV-specific bands could be visualized.

ALVAC immunoblot assay

ALVAC immunoblots were generated similar to that described for FIV immunoblots, using ALVAC derived from clarified lysates of ALVAC-infected primary chicken embryo fibroblasts. Serum samples obtained from ALVAC immunized animals and control non-immunized animals were tested at a serum dilution of 1:100 in Buffer 3. Reaction were carried out as described (see FIV immunoblot assay).

Enzyme-linked immunosorbent assays (ELISA)

Synthetic peptides corresponding to both conserved and variable regions in the FIV_{Pet} envelope surface(SU) and transmembrane(TM) protein were coated on 96 well Immunolon microtiter plates at 250 ng/well with bicarbonate buffer (pH 9.6).[V3-1 (SKWEEAKVKFHCQRTQSQPGS), V3-2 (GSWFRAISSWKQRNRWEWRDF), V3-3 (DFESKKVKISLQCNSTKNLFA) and TM (QLEGCNQNQFFCKI)]. The plates were washed with Buffer 3 immediately prior to use and blocked with 5% dry non-fat milk in H_2O. Serum samples were diluted 1:200 in Buffer 3 containing 5% newborn-calf serum and incubated in the coated wells for 30 min at 37°C, washed 3 times with ELISA wash solution (0.05 % Tween-20 in 0.15M sodium chloride), and incubated with biotinylated anti-cat IgG (Vector laboratories, Burlingame, CA) for 30 min at 37 °C. Subsequently, the wells were washed three times and incubated with streptavidin conjugated to horse radish peroxidase (Vector laboratories, Burlingame, CA), washed 3 times with ELISA wash

solution, followed by incubation with ELISA substrate solution (0.005 % tetramethylbenzidine and 0.015 % H_2O_2 in 0.96 % citrate solution). The reactions were stopped with 0.1 M sulfuric acid upon establishment of visible reaction color. The plates were read in a ELISA reader at 414 nm.

Assessment of viral neutralizing (VN) antibodies

The presence of FIV specific VN antibodies was evaluated using a standard assay (Yamamoto et al. 1991). Serum samples obtained preimmunization, postimmunization and after challenge were diluted at various concentrations (1:5 to 1:100) and incubated at 56°C for 30 minutes to inactivate complement. The diluted sera were then incubated with 100 $TCID_{50}$ (tissue culture cell infective doses of FIV_{Pet}) for 45 min at 37°C in a 24-well microtiter plate. Subsequently, peripheral blood mononuclear cells (PBMC) were added to this mixture at $1x10^6$ cells/well. After three days of culturing, cells were washed to remove residual virus from the culture and resuspended in fresh culture media (see RT media). Virus infection was monitored by Mg^{2+} dependent reverse transcriptase (RT) activity (see RT assay) in culture fluid harvested on Day 6, 9, 12, 15 and 18 of culturing. The VN antibodies titers were defined as the reciprocal of the highest final dilution which gave $\geq$50% reduction in reverse transcriptase activity as compared to the reverse transcriptase activity detected in fluids from control cell cultures that contained SPF serum and virus.

Assessment of FIV-specific proliferative T-cell responses

FIV-specific proliferative responses were evaluated using a ^{3}[H]-thymidine incorporation assay (Yamamoto et al. 1991). Freshly isolated PBMC were cultured in 96-well microtiter plates in a final volume of 200 µl in RPMI1640 media supplemented with 5% heat inactivated fetal calf serum, 10mM HEPES buffer, 50 mg/ml gentamycin, $5X10^{-5}$

M 2-mercaptoethanol at a final concentration of $1x10^6$ cells/ml ($2x10^5$cells/well). Triplicate cultures were stimulated with inactivated FIV (5 μg/well) and incubated at 37°C for 4 days in a humidified atmosphere containing 5% CO_2. On Day 4, cells were pulsed with 1 μCi ^{3}[H]-thymidine (Amersham, Indianapolis, IN) per well for 18 h. Cells were harvested onto filter paper using a cell harvester. The discs were air-dried and ^{3}[H]-thymidine incorporation was assessed by liquid scintillation counting. Results of triplicate samples were expressed as the stimulation index (S.I.), calculated as the mean incorporation in the presence of inactivated FIV divided by the mean incorporation in the absence of inactivated FIV.

Assessment of cytotoxic T-lymphocyte (CTL) responses

PBMC were tested for their ability to lyse autologous lymphoblastoid cells infected with FIV_{Pet}. Freshly isolated PBMC were cultured at $2x10^6$ cells/ml in RPMI1640 medium containing 10% FBS and stimulated with Con A (5 mg/ml) for three days. On Day 3, 3.6 x 10^6 Con A lymphoblasts were removed, infected with FIV_{Pet} and cultured for 5 days. The remaining cells (effector cells) were maintained in RPMI1640 medium supplemented with 10% FBS and IL-2 (100 U/ml) for 5 days. After 5 days, $1-5x10^6$ of the FIV-infected cells were inactivated by UV treatment and added as antigen-presenting cells(APC) to the effector cells at a ratio of 1:15 (APC:effector cells). Effector cells and FIV-infected APC cells were cocultured for an additional 5-7 days in no IL-2 medium. Effector cells were assayed for cytolytic activity against autologous FIV-infected target cells by a standard ^{51}Cr release assay (Song et al. 1992). Target cells were labeled with ^{51}Cr for 2 h at 37°C and washed three times prior to use in the assay. Effector and ^{51}Cr -labeled target cells were then mixed at effector to target ratios ranging from 50:1 to 10:1 and incubated for 4 h at 37°C without IL-2. After 4 h, 100 μl of supernatant was removed from each well and ^{51}Cr-specific activity was measured in a γ-counter. Results are shown as the percentage of

specific cytotoxicity for triplicate assays. Maximum release was obtained by repeated freeze-thawing of labeled target cells. Spontaneous release was obtained from ^{51}Cr labeled target cell cultures in the absence of effector cells. The percentage of FIV-specific release was calculated as 100 x (mean cpm test release - mean spontaneous release)/(mean cpm maximum release - mean cpm spontaneous release). The spontaneous release did not exceed 20% of the maximum release. Specific lysis values equal or greater than 10% were considered positive for CTL activity.

Assessment of natural killer cell activity

The level of natural killer (NK) cell activity was determined using a 4 h ^{51}Cr release assay similar to that described for the CTL assay. Various target cell types were used including FeT-J (feline lymphoid cell line), FL-4 (feline lymphoid cell line chronically infected with FIV_{Pet}), non-autologous PBMC, and autologous PBMC. Target cells were labeled with ^{51}Cr for 2 h at 37°C and washed three times prior to use in the assay. Freshly isolated PBMC effector cells were cocultured at effector to target cell ratios ranging between 100:1 to 10:1 for 4 h at 37°C in a humidified atmosphere containing 5% CO_2. After 4 h, 100 µl of supernatant was removed from each well and ^{51}Cr-specific activity was measured in a γ-counter. Results are shown as the percentage of cytotoxicity for triplicate assays. Maximum release was obtained by repeated freeze-thawing of labeled target cells. Spontaneous release was obtained from ^{51}Cr labeled target cell cultures in the absence of effector cells. The percentage of NK activity was calculated as 100 x (mean cpm test release - mean cpm spontaneous release)/ (mean cpm maximum release - mean cpm spontaneous release). The spontaneous release did not exceed 20% of the maximum release.

Viral reverse transcriptase (RT) assay

Freshly isolated PBMC were either stimulated with ConA (5mg/ml) or co-cultured and cultured for 4 weeks in RPMI1640 medium containing 5% heat inactivated FCS, 10mM HEPES buffer, 50 mg/ml gentamycin, $5X10^{-5}$ M 2-mercaptoethanol and 100 U/ml human recombinant IL-2, or cocultured with ConA lymphoblasts from SPF cats. Culture supernatants were collected every 3-4 days and assayed for the presence of viral reverse transcriptase (RT) activity. The virus was pelleted from the supernatants by ultra-centrifugation (1 h at 17,000rpm). The virus pellet was then incubated with an RT cocktail containing 100 mM Tris (pH8.3), 150 mM KCl, 10 mM $MgCl_2$, 4 mM diethiothreitol (DTT), 0.6 Units of Poly (rA), oligo(dT), and 60 μCi of ^{3}[H]TTP per ml. After incubation at 37°C for 1h, the cDNA was spotted onto filter paper discs that had been prewashed with 0.1 M sodium pyrophosphate. Discs were washed in the following sequence: twice in 10% cold trichloroacetic acid (TCA), once in 5% TCA, once in 5% TCA containing 0.5 % SDS, and once in ethanol. The filter discs were air-dried and placed in scintillation vials with 3 ml scintillation fluid. ^{3}[H]-TTP incorporation was measured in using a liquid scintillation counter. Supernatants were considered positive for RT activity if cpm in test samples were equivalent or higher than 3 times the cpm of the negative control sample (supernatants from SPF control cats).

Detection of proviral DNA by polymerase chain reaction (PCR)

Proviral DNA (latent infection) was monitored by *env* specific PCR on DNA extracted from PBMC, bone marrow (BM) cells, and lymph node (LN) cells after culturing for 4 weeks with FIV-free ConA lymphoblasts. BM cells were obtained from 1-2 ml of aspirates taken from the femur. LN cells were obtained from the popliteal lymph nodes.

DNA was extracted as described. In the PCR reaction, the following FIV *env* specific primer sets were used: 5'-GAAATGTATAATATTGCTGG-'3 and 5'-GAATTGATTTTGATTACATCC-'3. The PCR reactions were carried out in 50 μl reaction mixtures containing 1.0 μg genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dTTP and dGTP), 20 pmol of each primer, and 2.5 units of *Taq* DNA polymerase. The reaction mixture was incubated for 5 min at 94°C and cycled 30 times through 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min, followed by 10 min at 72°C. The specificity of the PCR-amplified 455bp product was verified by nucleotide sequence analysis.

DNA sequencing

DNA sequencing was performed using the Amplicycle sequencing kit (Perkin Elmer, Norwalk, CT). Primers (approximately 10 μM) were labeled at the 5'-end with 20 μCi γ-^{32}P ATP (6000Ci/mmol) and 20 U Polynucleotide kinase in a final reaction volume of 6.2 μl for 10 min at 37°C. The reactions were terminated by incubation at 90°C for 5 min. For the sequencing reactions, four separate reaction mixture were prepared containing: 1 μl labeled primer, 4 μl 10X reaction buffer and approximately 10-50 fmol of PCR template and 2 μl of either G, A, T, or C termination mix. Reaction mixtures were then incubated at 95°C for 15 seconds, cycled 25 times at 95°C for 1 min, 68°C for 1 min, followed by 7 min at 78°C. Upon cycling, 4 μl of formamide stop solution containing 0.05% bromophenol blue and xylene cyanol were added. The reactions were analyzed on a 6% polyacrylamide gel containing 7 M urea and 1X TBE.

In vivo assessment of viral-status

Three SPF kittens (#DH2, #DH5, #DE4), 12 weeks of age, were transfused intravenously with a total of 1.1 x 10^8 cells obtained 8 months after the FIV_{Pet} challenge from either ALVAC-*gag/prot* immunized cats (#QQ1 and #QX3) or FIV-infected ALVAC-control cat (#PY2). Cells consisted of 3 x 10^7 of PBMC isolated by ficoll hypaque density centrifugation, 7 x 10^7 BM cells and 1x10^7 LN cells. Prior to transfusion, these cells were washed in sterile PBS and resuspended in 2 ml PBS. Cat #DH2 received cells from infected control cat #PY2, cat #DH5 received cells from ALVAC-*gag/prot* immunized cat #QQ1 and cat #DE4 received cells from ALVAC-*gag/prot* immunized cat #QX3.

General parameters

Throughout the trial, all cats were monitored for hematological changes (complete blood count, differential leukocyte count and total protein count) and abnormal clinical manifestations (diarrhea, vomiting, lymphadenopathy, weight loss, elevated rectal temperature and neurological signs).

CD4/CD8 ratios

CD4/CD8 ratios were determined by indirect immunofluorescence staining and flow cytometry. Briefly, 5X10^5 PBMC isolated by ficoll hypaque density centrifugation, were washed in FACS buffer (PBS containing 0.325% sodium azide and 2.5% BSA) and incubated with feline CD4 or CD8 specific monclonal antibodies at 37°C for 1 h. Subsequently, cells were washed in FACS buffer and incubated with secondary antibody, FITC labeled goat $F(ab')_2$ anti-mouse IgG (H+L) (Southern Biotechnology Associates, Inc.) for 1 h at 37°C. Finally, cells were washed and analyzed by flow cytometry on a

Becton Dickinson FACSSORT. Monoclonal antibodies to feline CD4 and CD8 were kindly provided by N. Gengozian, University of Tennessee.

T-cell mitogen proliferative responses

T-cell mitogen proliferative responses were measured by ^{3}[H]-thymidine uptake assays (Ackley et al. 1990). Freshly isolated PBMC were resuspended at $2x10^{5}$ cells/ml and stimulated with either Con A (5 mg/ml) or SEA (1 mg/ml). Cells were cultured for 48 h and then pulsed with 1μCi ^{3}[H]-thymidine/well. After 18 h, cells were harvested using a cell harvester onto filter paper discs. The filter discs were air-dried and ^{3}[H]-thymidine incorporation was assessed by liquid scintillation counting. The results were expressed as the stimulation index (S.I.), calculated as incorporation (mean cpm of triplicate samples) in the presence of mitogen divided by incorporation (mean cpm of triplicate samples) in the absence of mitogen.

Statistical analysis

The statistical significance of the data was evaluated by a Fisher's exact test, which is a modification of the chi square test. This test should be used when comparing two sets of discontinuous, quantal (all or none) data. The analysis was set up as follows:

	Vaccinated	Unvaccinated
Infected	A	B
Uninfected	C	D

The P (Probability) for a one-tailed test was calculated as (A+B)!(C+D)!(A+C)!(B+D)! / N!A!B!C!D! combined with the P value of stronger combinations. The obtained P value tells if the groups differ significantly and the degree of significance. In this study, a P-value equal or less than 0.05 was considered significant.

Results

In vitro assessment

The ability of ALVAC-recombinants to infect non-permissive feline cells was demonstrated by PCR analysis on DNA extracted from feline CrFK cells inoculated with ALVAC-*env* and ALVAC-*gag/prot* recombinants (Figure 2.2 and 2.3). The obtained PCR products corresponded in size to PCR-products obtained with DNA extracted from FL-4 cells, a lymphoid cell line chronically infected with FIV_{Pet}. The correct nucleotide sequence was verified by DNA sequencing (data not shown). No FIV-specific PCR products could be detected in CrFK cells infected with ALVAC vector alone or FeT-J, a FIV-negative feline lymphoid cell line.

Similarly, the expression of messenger RNA (mRNA) specific for FIV*env* and *gag* products was demonstrated in CrFK cells inoculated with the ALVAC-*env* and ALVAC-*gag/prot* recombinants (Figure 2.4). RT-PCR on mRNA extracted from CrFK cells infected with ALVAC-*env* revealed a 450bp band and from those infected with ALVAC-*gag/prot* revealed a 700bp band consistent with the bands obtained from the FIV-infected FL-4 cell line. No RT-PCR products could be detected in cells infected with ALVAC vector alone and FeT-J control cells (FIV negative feline lymphoid cell line). In addition, no PCR products were obtained PCR reactions in which mRNA extracts were used as template, indicating that the obtained RT-PCR products were not the result of DNA contamination of mRNA extracts (data not shown).

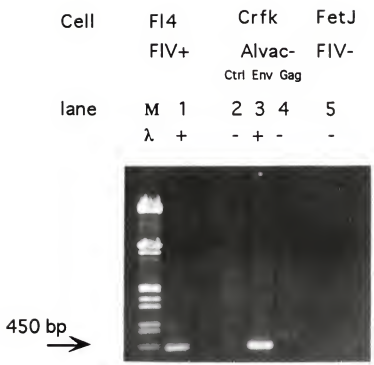


Figure 2.2 FIV-*env* specific PCR on CrFK inoculated with ALVAC-recombinants. Photograph of PCR products following electrophoresis on a 1.0 % agarose gel in Tris acetate-buffer (40mM Tris-acetate, 1 mM EDTA, pH 7.6). Lane M, λ DNA *EcoRI/HindIII* marker (1μg/lane). Lane 1, positive control, FIV-*env* specific PCR on DNA extracted from FL4 (FIV$^+$) cells. Lane 2, FIV-Env specific PCR on CrFK cells inoculated with ALVAC-control. Lane 3, FIV-*env* specific PCR on CrFK cells infected with ALVAC-*env*. Lane 4, FIV-*env* specific PCR on CrFK cells infected with ALVAC-*gag/prot*. Lane 5, negative control, FIV-Env specific PCR on Fet-J (FIV$^-$)cells.

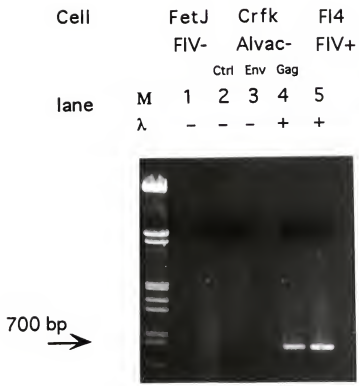


Figure 2.3 FIV-*gag* specific PCR on CrFK inoculated with ALVAC-recombinants. Photograph of PCR products following electrophoresis on a 1.0 % agarose gel in Tris acetate-buffer (40mM Tris-acetate, 1 mM EDTA, pH 7.6). Lane M, marker *EcoRI/HindIII* cut DNA (1 µg). Lane 1, negative control, FIV-*gag* specific PCR on DNA extracted from Fet-J (FIV⁻) cells. Lane 2, FIV-*gag* specific PCR on CrFK cells inoculated with ALVAC-control. Lane 3, FIV-*gag* specific PCR on CrFK cells infected with ALVAC-*env*. Lane 4, FIV-*gag* specific PCR on CrFK cells infected with ALVAC-*gag/prot*.. Lane 5, positive control, FIV-*gag* specific PCR on FI4 (FIV⁺)cells.

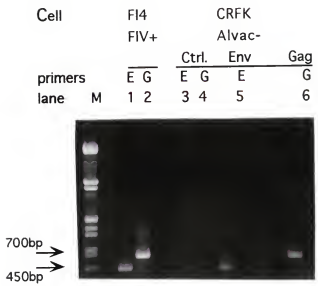
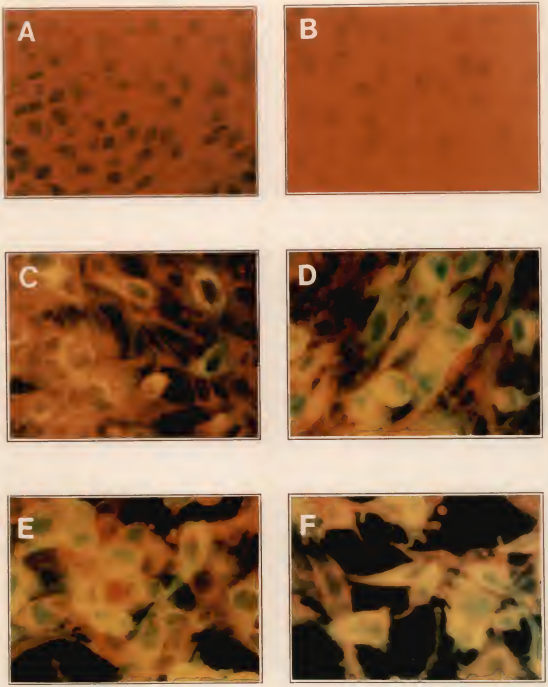


Figure 2.4 RT-PCR on mRNA extracted from CrFK inoculated with ALVAC-recombinants. Photograph of PCR products following electrophoresis on a 1.0 % agarose gel in Tris acetate-buffer (40mM Tris-acetate, 1 mM EDTA, pH 7.6). Lane M, λ DNA *EcoRI/HindIII* marker (1μg/lane). RT-PCR on mRNA extracted from FIV^{+} FL-4 cells shows specific amplification of FIV-*env* (Lane 1) and FIV-*gag* (Lane 2). RT-PCR on mRNA extracted from CrFK cells infected with ALVAC-control show no amplification of FIV-*env* (Lane 3) or FIV-*gag* (Lane 4). Lane 5, FIV-*env* specific RT-PCR on mRNA extracted from CrFK cells infected with ALVAC-*env*. Lane 6, FIV-*gag* specific RT-PCR on mRNA from CrFK cells infected with ALVAC-*gag/prot*.

Figure 2.5 Indirect immunofluorescence analysis on permeabilized CrFK cells infected with ALVAC-recombinants at a m.o.i. of 10. Expression of FIV Env glycoprotein and Gag proteins was detected using pooled serum from FIV-infected cats as the primary antibody and fluorescein isothiocyanate-conjugated mouse anti-cat IgG as the secondary antibody. Panel A, control uninfected CrFK cells. Panel B, control CrFK cells infected with ALVAC-control. Panel C, CrFK cells infected with ALVAC-*env*. Panel D, CrFK cells infected with ALVAC-*gag/prot*. Panel E, CrFK cells infected with ALVAC-*env,gag/prot*. Panel F, CrFK cells infected with ALVAC-*97TMG*.


A
B
C
D
E
F

Further, the expression of the ALVAC encoded FIV Env as a membrane associated protein and of the FIV-Gag core protein as intracellular protein was demonstrated by indirect immunofluorescence on CrFK cells inoculated with the ALVAC-FIV recombinants (Figure 2.5). At 48 h postinfection, cells infected with ALVAC-*env* showed fluorescence predominantly at the surface (panel C) whereas cells infected with ALVAC-*gag/prot*, ALVAC-*env,gag/prot* and ALVAC-*97TMG* showed strong fluorescence in the cytoplasm (see Figure 2.5, panel D-F). Control cells, uninfected CrFK and CrFK infected with ALVAC vector alone, did not show immunofluorescence (panel A and B).

In vivo assessment

A total of 36 SPF cats were used to evaluate the prophylactic efficacy of immunizations protocols employing ALVAC-FIV recombinants alone or in combination with ICV. Cats were divided into 7 groups (A-G) and inoculated three times at monthly intervals with either ALVAC-*env* (n=6), ALVAC-*gag/prot* (n=6), ALVAC-*env,gag/prot* (n=6), ALVAC-*97TMG* (n=6) or ALVAC vector alone (n=6). Cats in group F and G were immunized twice with ALVAC-*env,gag/prot* (n=3) or ALVAC (n=3), respectively, followed by a boost with inactivated FIV-infected cell vaccine (see Table 2.1). All cats were challenged 4 weeks after the final immunization with 50 ID_{50} FIV_{Pet}. The FIV_{Pet} isolate is a subtype A virus, and differs 3% in the Env and 1% in the Gag protein coding region from the $FIV_{Ville\ franche}$ isolate (subtype A) used to generate the ALVAC recombinants.

General immunologic parameters

No adverse effects and no significant changes in blood chemistry (CBC, HgB, PCV, TPP) were noticed in any of the cats upon immunization with ALVAC-FIV recombinants or after boosting with inactivated FIV-infected cell vaccine (data not shown).

Figure 2.6 Representative FACS analysis of binding of anti-fCD4 and anti-fCD8 monoclonal antibodies to PBMC. Expression of fCD-4 and fCD-8 was detected by using anti-fCD4 and anti-fCD8 antibodies as the primary antibody and fluorescein isothiocyanate-conjugated goat anti-mouse IgG as the secondary antibody. Panel A, scattered dot-plot of PBMC isolated from cat #QG3. The depicted histograms respresents cells gated under gate 1 (predominantly lymphocytes) and gate 2 (negative control, cells of the macrophage and monocyte lineages). Panel B, histogram of CD4 and CD8 staining of cells in gate 1. Panel C, histogram of CD4 and CD8 staining of cells in gate 2.

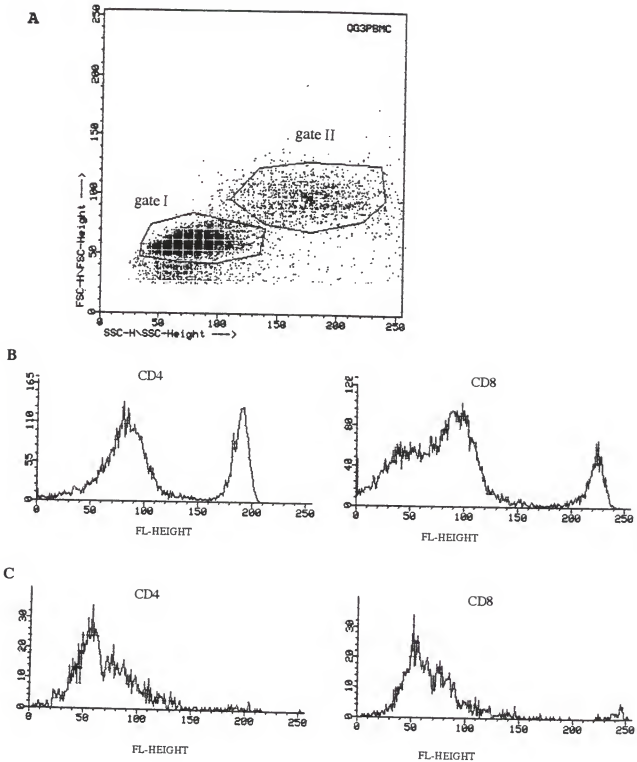

A
003PBMC
gate II
gate I
FSC-H\FSC-Height ⟶
SSC-H\SSC-Height ⟶
B
CD4
CD8
FL-HEIGHT
FL-HEIGHT
C
CD4
CD8
FL-HEIGHT
FL-HEIGHT

Table 2.2 CD4/CD8 ratios before and after immunization and challenge

Cat ID#	Immunizations Before -4	Immunizations After -1	months post-challenge +3	+5	+6	+7	+8	+10	+12
QH4	4.7	4.4		3.2	3.0	3.3^{F}			
PY1	4.3	4.2		2.4	2.4	2.5^{F}			
QO1	3.9	2.6	3.5^{F}						
QC1	3.3	3.3		2.9	2.5		1.9^{F}		
QU1	ND	3.8		3.6	3.3		2.5^{F}		
QL2	3.6	3.3		2.2	1.9	1.7^{F}			
QH5	6.7	4.2		3.8	2.6		2.5^{F}		
PY3	5.1	4.9		4.3	3.3		2.6^{F}		
QS4	6.0	4.8		2.6	2.3	2.3^{F}			
QC3	3.2	2.6		3.6	2.1		2.0^{F}		
QG3	5.0	4.0		3.6	3.2		2.4^{F}		
QE2	3.1	ND		3.0	2.0^{F}				
QQ1	4.9	2.7		2.1	1.9			1.6	2.3
QA5	4.3	2.2		2.6	2.5			2.9	2.5
QU2	4.0	2.6		2.4	2.0			2.3	2.9
QX3	2.0	2.5		2.0	1.8			1.8	2.3
QI1	6.1	3.9		3.9	3.3			3.3	3.9
QL3	4.9	2.0		2.6	1.9			2.0	2.2
QQ2	2.7	3.4		2.7	2.0		2.2^{F}		
PY5	5.1	3.8		2.5	2.1		1.9^{F}		
QO2	3.3	4.0	2.4^{F}						
QX4	1.3	ND		1.5	1.1	1.0^{F}			
QI2	4.9	2.5		2.1	2.0	1.9^{F}			
QL4	4.4	4.1		3.8	2.3		2.5^{F}		
QH2	3.9	3.5	4.6^{F}						
PY2	3.3	2.9		3.5	2.7			3.8	2.5
QA4	2.9	2.6		2.4	1.7		1.8^{F}		
QC4	4.2	3.1		2.7	2.4	1.6^{F}			
QG5	4.0	2.5	2.7^{F}						
QE3	2.1	2.3		2.7	1.7		2.0^{F}		
QH3	5.5	4.1		4.0	3.4			2.0	4.3
PY4	2.8	4.0		3.1	2.6			2.6	2.4
QA6	4.3	3.6		2.9	2.5			2.1	2.7
QC5	2.8	2.0	2.1^{F}						
QG4	5.1	3.5	2.9^{F}						
QE4	2.6	3.1		1.8	2.0			1.6	2.5

F=Final testing of CD4/CD8 ratio before animal was euthanized.

Table 2.3 Proliferation responses to T-cell mitogens (ConA and SEA).

Vaccine	Cat ID#	Stimulation Index							
		Number of immunizations							
		Pre-Immunization		1X		3X		Post-Challenge[a]	
		ConA	SEA	ConA	SEA	ConA	SEA	ConA	SEA
ALVAC-*env*	QH4	2.8	26.7	7.9	17.4	22.5	24.9	2.5	3.1
	PY1	4.3	29.0	6.6	12.6	4.0	4.0	3.2	3.1
ALVAC-*gag/prot*	QA5	7.5	16.9	32.1	77.4	30.3	14.4	22.7	23.2
	QI1	50	93.8	2.2	7.5	25.8	28.2	18.3	17.8
ALVAC-*env*, *gag/prot*	QS4	6	29	3.1	6.7	35.0	35.2	37.1	36.7
	QG3	2.9	6.9	1.0	2.3	38.1	35.0	30.7	22.7
ALVAC-*97TMG*	QQ2	2.7	9.2	1.0	2.0	16.8	18.0	17.2	12.5
	QL4	78.4	99.0	9.6	20.3	29.4	27.2	18.8	17.2
ALVAC-*env*, *gag/prot* &ICV	PY4	13.4	26.8	4.0	15.9	20.5	19.7	63.2	36.7
ALVAC	QH2	4.2	7.2	2.4	5.5	9.5	9	28.7	26.2
	QA4	46	65	5.5	9.3	44.3	52.8	15.2	17.3
ALVAC &ICV	QG4	4	8	12.5	23.5	7.4	7.4	25.4	23.5

[a] 3 weeks post challenge

CD4/CD8 ratios were monitored prior to immunization, postimmunization and after challenge, a representation of a typical FACS analysis is depicted in Figure 2.6. Prior to immunization, most cats displayed CD4/CD8 ratios considered normal for three month old kittens (average ratio 4.0 $\pm$ 1.2) except for cat #QX3 which displayed a ratio of 1.3 which is considered low. A littermate of this cat (#QX4) also displayed a relatively low ratio, suggesting that genetic factors attributed to this. After immunization, most cats showed an average CD4/CD8 ratio of 3.3 $\pm$ 0.8. The observed decline in ratio is expected with the increase in age. No inversion (<1) in CD4/CD8 ratios were noticed after challenge (Table 2.2). This was as expected since the FIV_{Pet} at the challenge dose used generally does not cause inversion of CD4/CD8 ratio until 1.5-2 years postchallenge.

To determine if ALVAC vaccinations influenced lymphocyte function, we evaluated lymphocyte proliferation upon exposure to concanavalin A (ConA) and staphylococcal enterotoxin A (SEA) at various times in selected cats. Following immunizations and challenge no abnormalities in T-cell proliferative responses were detected in any of the cats tested (Table 2.3).

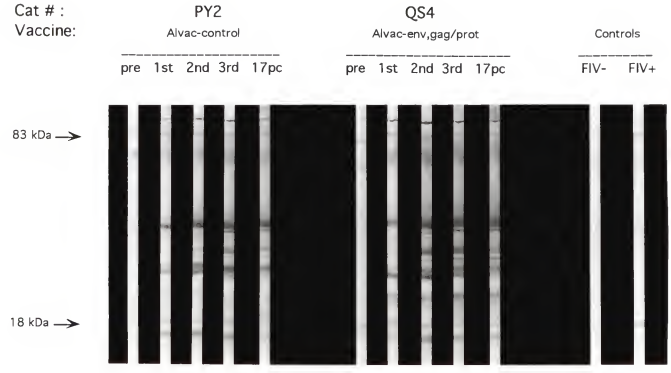
Humoral responses

The generation of ALVAC-specific antibodies was evaluated before and after immunization in serum samples taken from cat #QS4 immunized with ALVAC-*env,gag/prot* and cat #PY2 immunized with ALVAC vector alone by immunoblotting. ALVAC specific humoral responses were detected in both cats upon a single immunization and additional immunizations resulted in increased titers of these antibodies (Figure 2.7). Serum samples obtained from SPF and FIV infected control cats tested negative.

Next, we tested if the immunization schemes used were able to generate FIV-specific antibody responses. Immunization with ALVAC-FIV recombinants alone (#QC3) failed to induce detectable FIV-specific antibody responses even after three immunizations

Figure 2.7 ALVAC-specific immunoblot. Serum samples taken from ALVAC-control immunized cat #PY2 and ALVAC-*env,gag/prot* immunized cat #QS4 prior to immunization (pre-) and after the first, second and third immunization and 17 weeks postchallenge (17pc) were diluted 1:100 in Buffer 3 and incubated with ALVAC-specific westernblot strips. Negative controls: pooled sera from FIV-infected cats and pooled sera from FIV-negative SPF cats.

Immunoblot ALVAC



serum dilution: 1:100

Figure 2.8 FIV-specific immunoblot. Serum samples from ALVAC-*env,gag/prot* immunized cat #QC3, ALVAC-*env,gag/prot* combined with ICV immunized cat #QA6 and ALVAC combined with ICV immunized cat #QC5, obtained before and after immunizations, were diluted 1 to 100 in Buffer 3 and incubated with FIV-specific westernblot strips. The positive control was incubated with pooled serum from FIV-infected cats. The negative control was incubated with serum from a SPF cat.

Immunoblot FIV

Cat #	QC3	QA6	QC5	
Vaccine	Alvac-Env,Gag/pro(3X)	Alvac-Env,Gag/pro(2X) ICV(1X)	Alvac-control(2X) ICV(1X)	Controls
	pre 1st 2nd 3rd	pre 1st 2nd 3rd	pre 1st 2nd 3rd	+ -

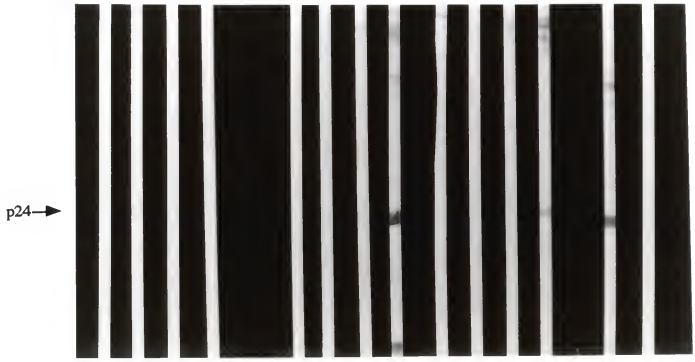


Table 2.4 FIV-specific antibody titers before and after immunizations and challenge.

Cat ID#	Vaccine	Boost	FIV-specific antibody titer[a]			
			pre-immunizations	post	Mo. postchallenge 2	8
QA6	ALVAC-*env,gag/prot*	ICV(1X)	<2	5-6	4-5	4-5
QH3	ALVAC-*env,gag/prot*	ICV(1X)	<2	5	3-4	4
PY4	ALVAC-*env,gag/prot*	ICV(1X)	<2	5	4-5	4-5
QG4	ALVAC(2X)	ICV(1X)	<2	4-5	5	ND
QC5	ALVAC(2X)	ICV(1X)	<2	3-4	5-6	ND
QE4	ALVAC(2X)	ICV(1X)	<2	3-4	5-6	5-6
QS4	ALVAC-*env,gag/prot* (3X)		<2	<2	5-6	ND
QC3	ALVAC-*env,gag/prot* (3X)		<2	<2	<2	ND
PY3	ALVAC-*env,gag/prot* (3X)		<2	<2	<2	ND
PY2	ALVAC(3X)		<2	<2	5-6	5-6

[a] FIV-specific titers expressed as the reciprocal of the highest dilution (in ^{10}log) at which FIV specific bands could be detected by immunoblotting.
ND= not determined

(Figure 2.8). In contrast, all cats boosted with ICV (group F and G) developed detectable FIV-specific antibody responses. Interestingly, cats primed with ALVAC-*env,gag/prot* (#QA6) developed approximately 10-fold higher antibody titers than those primed with ALVAC vector alone (#QC5) (Figure 2.8) upon the ICV boost (see Table 2.4)

Selected cats of each group were also tested for the presence of antibody responses to peptides corresponding to the V3 region of the FIV surface envelope glycoprotein by ELISA. This region is thought to be equivalent to the V3 region of HIV which contains the principal neutralizing domain (Pancino et al. 1994). None of the immunized cats exhibited significant levels of antibody titers to the three V3 peptides tested even after three immunizations (data not shown).

Viral neutralizing antibody responses were measured in selected cats before and after immunization. The neutralization assay was performed using ConA lymphoblast as FIV-susceptible cells and FIV_{Pet} propagated on a feline lymphoid cell line (FL-4) as the virus inoculum. VN antibody responses were absent before immunization and after immunization in all cats tested including those boosted with ICV (Table 2.5).

Table 2.5 Viral neutralizing antibody titers after immunization and challenge

Vaccine	Cat ID#	VN titer[a] pre-immunizations	post-immunizations	Months post-challenge 3	12
ALVAC-*env*	QU1	<5	<5	<5	NT
	PY1	<5	<5	>100	NT
ALVAC-*gag/prot*	QX3	<5	<5	<5	<5
	QQ1	<5	<5	<5	<5
	QI1	<5	<5	<5	<5
	QL3	<5	<5	<5	<5
ALVAC-*env,gag/prot*	QS4	<5	<5	>100	NT
	PY3	<5	<5	<5	NT
ALVAC-*env,gag/prot* &ICV	QH3	<5	<5	5-20	<5
	QA6	<5	<5	5-20	<5
	PY4	<5	<5	5-20	5-20
ALVAC	QC4	<5	<5	>100	NT
	PY2	<5	<5	>100	>100
	QA4	<5	<5	<5	NT
	QE3	<5	<5	<5	NT
ALVAC &ICV	QG4	<5	<5	>100	NT
	QC5	<5	<5	>100	NT
	QE4	<5	<5	>100	>100

[a] VN Titer expressed as the reciprocal of the highest final dilution which gave $\geq$ 50% reduction in reverse transcriptase activity as compared to reverse transcriptase activity observed in control cultures which contained SPF serum. NT= not tested.

T-helper Lymphoproliferative Responses

Selected cats from each group were tested after the second and third immunization and challenge for lymphocyte proliferation in response to inactivated FIV (Table 2.6). The inactivated FIV preparation used in this assay, included significant amounts of Gag allowing the detection of proliferative responses to both FIV Env and Gag. After two immunizations, no significant levels of FIV-specific lymphoproliferative responses were detected in any of the tested cats. After the third immunization lymphoproliferative responses were detected in two cats. However, the observed levels were low as compared to those detected in cats immunized multiple times with ICV vaccines (S.I. 4-6). This included cat #QC3 immunized with ALVAC-*env,gag/prot* and cat #PY4 immunized with ALVAC-*env,gag/prot* and boosted with ICV. Upon challenge, lymphoproliferative responses were absent in all of the tested cats even in cats that became viremic except for cat #QX4 which showed low levels of FIV-specific proliferative responses (Table 2.6).

Cytotoxic T-cell responses

Since viral-vector based vaccines are thought in general to be effective in eliciting CTL responses, FIV-specific CTL responses were measured after each immunization in the peripheral blood of selected cats. PBMC isolated were cultured in the presence of FIV antigen-presenting cells and assayed for their ability to lyse autologous PBMCs infected with FIV_{Pet}. FIV-specific CTL activity was detected in one of two cats tested after a single immunization with ALVAC-*env,gag/prot* (see Table 2.7). After the second and third immunization, CTL activity was detected in some cats of each group immunized with ALVAC-FIV recombinants alone and those immunized with ALVAC-*env,gag/prot* and boosted with ICV. No major variance between the different immunization schemes, with respect to intensity of CTL activity or percentage of cats displaying CTL activity within

Table 2.6 T-helper responses to FIV upon immunization and challenge

Vaccine	Cat ID#	Stimulation Index		
		Number of Immunizations 1X	3X	Post-challenge[a]
ALVAC-*env*	QL2	1.1	1.2	ND
	QH4	ND	ND	1.0
	QC1	ND	ND	0.7
ALVAC-*gag/prot*	QX3	1.0	1.3	1.4
ALVAC-*env,gag/prot*	QG3	0.9	1.6	ND
	QC3	ND	2.6	1.3
	PY3	ND	1.6	ND
	QH5	ND	ND	0.7
ALVAC-*97TMG*	QI2	1.1	1.7	ND
	QX4	ND	ND	2.1
ALVAC	QC4	1.2	ND	ND
	QE3	ND	1.1	ND
	QH2	ND	ND	1.3
ALVAC-*env*, *gag/prot* &ICV	PY4	ND	2.4	ND
	QH3	ND	ND	0.7
ALVAC &ICV	QC5	0.7	1.3	0.7
	QG4	ND	1.0	ND

[a] 4 weeks post-challenge
ND= not determined.

Table 2.7 FIV specific CTL activity in peripheral blood after immunizations

Vaccine	Cat ID#	% specific ^{51}Cr release[a] Number of Immunizations 1X	2X	3X
ALVAC-*env*	QH4	ND	3.9	9.5
	QC1	ND	ND	22.9
	PY1	ND	ND	3.7
ALVAC-*gag/prot*	QQ1	ND	ND	22
	QA5	ND	ND	4
	QI1	ND	ND	8
ALVAC-*env,gag/prot*	QC3	0.1	ND	18.4
	PY3	25	9.9	36.7
	QS4	ND	22	7
	QH5	ND	ND	1
ALVAC-*97TMG*	QQ2	ND	25.6	19
	PY5	ND	ND	12.5
	QI2	ND	ND	9.0
ALVAC-*env,gag/prot* &ICV	QH3	ND	0.4	55.2
	QA6	ND	ND	27.3
	PY4	ND	ND	8.0
ALVAC&ICV	QG4	ND	ND	10
ALVAC	QA4	ND	ND	7
	QH2	ND	1.3	ND
	PY2	ND	ND	0.0
	QC4	ND	ND	10

[a] Percentage specific release as observed at an average effector to target cell ratio of 1 to 20-30.
ND= not determined

each group, was observed. Further, cats primed with ALVAC-*env,gag/prot* and boosted with ICV displayed similar levels of CTL activity as detected in those immunized with ALVAC-*env,gag/prot* alone. Control cats immunized with ALVAC vector alone or immunized with ALVAC (2X) and boosted with ICV failed to demonstrate significant levels of CTL activity (< 10%).

The detected CTL activity was found to be MHC restricted as effector cells were only capable of lysing autologous FIV-infected target cells and failed to lyse non-autologous FIV-infected target cells (data not shown), thus implying that the detected activity was due to CTL, as opposed to NK cell activity. Further, no lysis was observed using uninfected autologous target cells (data not shown).

Table 2.8 NK activity postchallenge

		% specific ^{51}Cr release			
		Target cell			
Vaccine	Cat ID#	autologous PBMC	heterologous PBMC	FL-4	Fet-J
ALVAC-*env*	QC1	0	0	10.1	20.7
ALVAC-*gag/prot*	QC3	ND	0	12.5	18.8
ALVAC-*env, gag/prot*	QX3	0	0	8.7	22.8
ALVAC-*97TMG*	QX4	ND	0	10.7	16.9
ALVAC &ICV	QC5	ND	0	5.5	17.8
ALVAC	QC4	ND	12.2	6.5	8.2

ND= not determined

NK cell activity

NK cells are the principal effector cells in clearance of viral infections early in the course of infection. For this reason, we evaluated levels of NK activity in selected cats at 3.5 weeks postinfection (Table 2.8). NK activity was measured using various target cells including autologous PBMC as negative controls. All cats displayed normal levels of NK activity and no significant differences were observed between the infected and non-infected ALVAC-immunized cats.

Protective efficacy

The presence or absence of FIV following challenge was measured at monthly intervals using several methods. This included the assessment of viral reverse transcriptase (RT) activity and FIV-specific PCR analysis of cultured PBMC. These assays were also used to test LN and BM cells for the presence of FIV, as these organs function as major reservoirs for the virus. In addition, FIV infection was determined by comparing the level of FIV-specific antibody responses in the serum before and after FIV challenge. FIV-specific immunoblotting (WB) was performed as well as ELISA using a peptide corresponding to the transmembrane (TM) region of the FIV Env. Sera from FIV_{Pet} infected cats have shown to react strongly to this peptide. Further, VN antibody responses were measured in selected cats before and after FIV challenge. In general, the induction and persistent elevation of FIV-specific antibody responses and VN antibodies are indicative of an active viral infection.

All control cats (n=3) immunized with ALVAC vector alone and boosted with ICV became viremic as assessed by RT for infectious virus and FIV-specific PCR for proviral DNA in peripheral blood and tissue samples (BM and LN cells) (Table 2.9b and Table 2.10). Further, these cats developed high titer VN (>100) responses indicative of active

Table 2.9a Virus isolation (RT and PCR) on PBMC and immunoblot analysis.

Vaccine	Cat ID#	Immunizations pre--3 WB RT PCR	Immunizations post--1 WB RT PCR	Months post-challenge +1 WB RT PCR	+2 WB RT PCR	+3 WB RT PCR	+4 WB RT PCR	+8 WB RT PCR	+12 WB RT PCR
	QH4	- - -	- - -	- - -	- - -	- - -	- - -		
	PY1	- - -	- - -	- + +	+ + +	+ + +	+ + +		
ALVAC-*env*	QO1	- - -	- - -	- + +	+ + +	+ + +			
	QC1	- - -	- - -	- - -	- - -	- - -	- - -		
	QU1	- - -	- - -	- - -	- - -	- - -	- - -		
	QL2	- - -	- - -	- + +	+ + +	+ + +	+ + +		
	QQ1	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
	QA5	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
ALVAC-*gag/prot*	QU2	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
	QX3	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
	QI1	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
	QL3	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
	QH5	- - -	- - -	- - -	- - -	- - -	- - -		
	PY3	- - -	- - -	- - -	- - -	- - -	- - -		
ALVAC-*env*,	QS4	- - -	- - -	- + +	+ + +	+ + +	+ + +		
gag/prot	QC3	- - -	- - -	- - -	- - -	- - -	- - -		
	QG3	- - -	- - -	- - -	- - -	- - -	- - -		
	QE2	- - -	- - -	- - -	+ - -	+ + +	+ - +		

WB= western blot (FIV-specific), RT= reverse transcriptase, PCR using FIV-specific primers.

Table 2.9b Virus isolation (RT and PCR) on PBMC and immunoblot analysis.

Vaccine	Cat ID#	Immunization pre--3 WB RT PCR	Immunization post--1 WB RT PCR	Months post-challenge +1 WB RT PCR	+2 WB RT PCR	+3 WB RT PCR	+4 WB RT PCR	+8 WB RT PCR	+12 WB RT PCR
	QQ2	- - -	- - -	- - -	- - -	- - -	- - -		
	PY5	- - -	- - -	- - -	- - -	- - -	- - -		
ALVAC-*97TMG*	QO2	- - -	- - -	- + +	+ + +	+ + +			
	QX4	- - -	- - -	- + +	+ + +	+ + +	+ - -		
	QI2	- - -	- - -	- - +	+ + +	+ + +	+ + +		
	QL4	- - -	- - -	- - -	- - -	- - -	- - -		
	QH2	- - -	- - -	- + +	+ + +	+ + +			
	PY2	- - -	- - -	- - -	+ + +	+ + +	+ + +	+ + +	+ + +
ALVAC	QA4	- - -	- - -	- - -	- - -	- - -	- - -		
	QC4	- - -	- - -	- - -	+ + +	+ + +	+ + +		
	QG5	- - -	- - -	- + +	+ + +	+ + +			
	QE3	- - -	- - -	- - -	- - -	- - -	- - -		
ALVAC-*env, gag/prot* &ICV	QH3	- - -	+ - -	+ - -	+ - -	+ - -	+ - -	+ - -	
	PY4	- - -	+ - -	+ - -	+ - -	+ - -	+ - -	+ - -	
	QA6	- - -	+ - -	+ - -	+ - -	+ - -	+ - -	+ - -	
ALVAC &ICV	QC5	- - -	+ - -	+ + +	+ + +	+ + +	+ + +		
	QG4	- - -	+ - -	+ + +	+ + +	+ + +	+ + +		
	QE4	- - -	+ - -	+ - -	+ + +	+ + +	+ + +	+ + +	+ + +

WB= western blot (FIV-specific), RT= reverse transcriptase, PCR using FIV-specific primers.

Table 2.9 Virus isolation on PBMC and tissue samples and WB and ELISA data

Vaccine	Cat#	Weeks p.c.		Tissue							
				PBMC		BM		LN		THY	
			WB/ELISA	RT	PCR	RT	PCR	RT	PCR	RT	PCR
	QH4	27	- -	-	+	-	+	-	+	-	-
	PY1	24	+ +	-	-	-	-	-	+	-	-
Alvac-*env*	QO1	10	+ +	+	+	+	+	+	+	ND	
	QC1	28	- -	-	-	-	-	-	-	ND	
	QU1	28	- -	-	-	-	-	-	-	ND	
	QL2	24	+ +	-	-	+	+	+	+	-	-
	QQ1	29	- -	-	-	-	-	-	-	ND	
	QA5	29	- -	-	-	-	-	-	-	ND	
Alvac-*gag/prot*	QU2	29	- -	-	-	-	-	-	-	ND	
	QX3	29	- -	-	-	-	-	-	-	ND	
	QI1	29	- -	-	-	-	-	-	-	ND	
	QL3	29	- -	-	-	-	-	-	-	ND	
	QH5	28	- -	-	-	-	-	-	-	-	-
	PY3	27	- -	-	-	-	-	-	-	ND	
Alvac-*env*,	QS4	24	+ +	-	-	-	+	+	+	-	-
gag,prot	QC3	28	- -	-	-	-	-	-	-	-	-
	QG3	28	- -	-	-	-	-	-	-	-	-
	QE2	28	+ +	-	-	-	+	-	+	-	+
	QQ2	27	- -	-	-	-	-	-	+	-	-
	PY5	28	- -	-	-	-	-	-	-	-	-
Alvac-*97TMG*	QO2	10	+ +	+	+	+	+	+	+	ND	
	QX4	25	+ +/-	-	-	-	-	-	-	-	-
	QI2	25	+ -	-	-	-	-	-	-	-	-
	QL4	27	- -	-	-	-	-	-	-	-	-
	QH2	10	+ +/-	+	+	+	+	+	+	ND	
	PY2	39	+ -	+	+	-	+	-	-	ND	
Alvac-*control*	QA4	28	- -	-	-	-	-	-	-	-	-
	QC4	26	+ +	+	+	+	+	+	+	+	+
	QG5	10	+ +	-	+	-	+	+	+	ND	
	QE3	28	- -	-	-	-	-	-	-	-	-
Alvac-*env*,	QH3	36	+ -	-	-	-	-	-	-	ND	
gag/prot&ICV	PY4	36	+ -	-	-	-	-	-	-	ND	
	QA6	36	+ -	-	-	-	-	-	-	ND	
Alvac-*control*	QC5	10	+ +	+	+	-	-	+	+	ND	
&ICV	QG4	10	+ +	-	+	-	+	+	+	ND	
	QE4	39	+ +	-	-	+	+	ND		ND	

WB=western blot, PBMC= peripheral blood mononuclear cells, BM= bone marrow, LN=lymph node, Thy=thymus, ND= not determined.

viral infection (Table 2.5). In contrast, only 4 of 6 control cats immunized with ALVAC vector alone became viremic as determined by RT, PCR and development of FIV-specific antibodies including VN antibodies. The two other cats (#QA4 and #QE3) in this group tested negative consistently by RT and PCR analysis in peripheral blood samples taken 1, 2, 3, 4 and 7 mo after challenge. In addition, BM, LN and thymus tissues taken from these two cats 7 mo after challenge were negative for virus by RT and PCR analysis (Table 2.9b). These cats also failed to develop FIV-specific antibody responses as assessed by immunoblot (WB) and ELISA and lacked detectable levels of VN antibody responses, further supporting lack of infection in these cats.

In the group immunized with ALVAC-*env* two of six cats tested negative for virus by RT, and PCR analysis at all time-points postchallenge (Table 2.9a). In addition, these cats failed to develop detectable levels of FIV-specific and VN antibody responses (Table 2.5). Another cat (#QH4) in this group also tested negative by all these criteria up to 6 mo postchallenge but tested positive for virus by PCR analysis in PBMC, BM and LN cells taken 7 mo postchallenge (Table 2.10). The three remaining cats in this group developed viremia upon challenge at a rate similar to that observed in the ALVAC control cats that became infected. Partial protection was also observed in the groups immunized with ALVAC-*env,gag/prot* and ALVAC-*97TMG*. Four out of 6 cats immunized with ALVAC-*env,gag/prot* and 3 out of 6 cats immunized with ALVAC-*97TMG* resisted infection following challenge. The remaining cats in these groups tested positive for virus by all criteria except for cat #QQ2 which tested positive by PCR analysis only in lymph node tissue taken 7 months after challenge (Table 2.9b and Table 2.10).

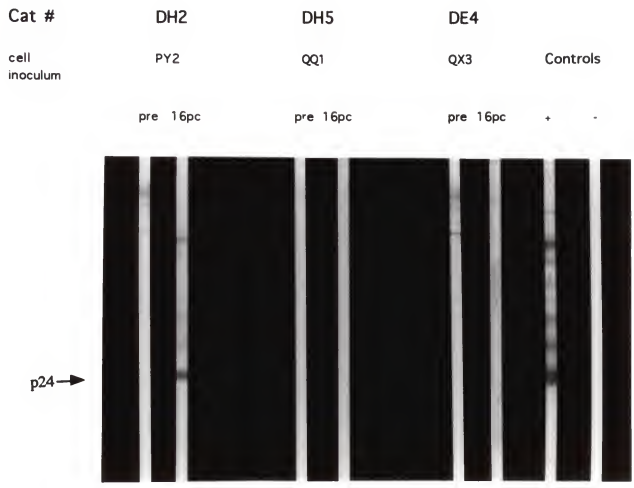
In contrast, full protection was observed in the group immunized with ALVAC-*gag/prot* . All six cats tested negative by RT and PCR in peripheral blood and lymphoid tissues. Further, these cats lacked detectable levels of FIV-specific antibodies and VN antibodies up to one year postchallenge (Table 2.5). Similarly, cats primed with ALVAC-*env,gag/prot* and boosted with ICV tested negative by RT and PCR analysis of peripheral

blood and lymphoid tissues throughout the vaccine trial (Table 2.9a and 2.10). FIV-specific humoral responses elicited by vaccination remained following challenge but slowly declined thereafter. Interestingly, serum samples obtained three months postchallenge contained low VN antibody titers (5-20) whereas no VN titers were detected in the sera taken from these cats after the third immunization. At 8 mo postchallenge, VN antibody titers persisted at low level in cat #PY4 and could no longer be detected in cat #QA6 and #QH3. The low titer of VN antibodies detected in this group as compared to those detected in infected control cats and the observed delay suggest that these responses were the result of anamnestic responses to the challenge inoculum rather than active viral infection.

In vivo transfer study

As an additional means of analyzing the viral status of ALVAC-*gag/prot* immunized cats after challenge, two naive SPF kittens were transfused with PBMC, BM and LN cells isolated from cat #QX3 and #QQ1 at 8 months postchallenge. An additional SPF kitten, which served as a positive control, was transfused with cells from FIV-infected control cat #PY2. Following challenge, kittens were monitored for viral infection at monthly intervals by methods analogous to those described previously. Kitten #DH2, which was transfused with cells from FIV_{Pet} infected cat #PY2, became readily infected as shown by RT, PCR (data not shown), and immunoblotting (Figure 2.9). In contrast, kittens #DH5 and #DE4 which were transfused with cells from ALVAC-*gag/prot* immunized cats, were negative for virus by RT and PCR up to 5 months after the transfusion in peripheral blood, LN and BM tissues (data not shown). Further, these cats failed to develop FIV-specific humoral responses as determined by immunoblotting (Figure 2.9).

Figure 2.9 FIV-specific immunoblot. Serum samples obtained before (pre) and 16 weeks (16pc) after transfusion from kittens #DH5 and #DE4, transfused with cells from ALVAC-*gag/prot* immunized cats, and cat #DH2 transfused with cells from ALVAC-control immunized cat, were diluted 1 to 100 in Buffer 3 and incubated with FIV-specific westernblot strips. The positive control was incubated with pooled serum from FIV-infected cats. The negative control was incubated with serum from a SPF cat.


Cat #
DH2
DH5
DE4
cell inoculum
PY2
QQ1
QX3
Controls
pre 16pc
pre 16pc
pre 16pc
+
-
p24

Statistical analysis

The Fisher's exact test was used to analyze the statistical significance of the protective efficacy data (Table 2.11). The infectivity rate of the ALVAC-FIV recombinant immunized groups was compared to that of the ALVAC (n=6) control group alone or to that of the ALVAC control group combined with the ALVAC/ICV (n=3) immunized control group (total n=9). The infectivity rate of ALVAC-*env,gag/prot*ICV immunized cats was compared to either the ALVAC/ICV-immunized control group (n=3) or to both the ALVAC-immunized and ALVAC/ICV-immunized control groups (n=9). Based on this test, immunization schemes employing ALVAC-*gag/prot* and ALVAC-*env,gag/prot* together with ICV, showed significant protection as indicated by a P (Probability) value equal or less than 0.05 (Table 2.11). Protection observed by the other immunization schemes was not significant (P value > 0.05).

Table 2.11 Statistical analysis

	Viral status			
Vaccine	vaccine group +/-	control group +/-	P value (single-tailed)	significant
ALVAC-*env*	3/3	4/2	0.5	no
	3/3	7/2	0.28	no
ALVAC-*gag/prot*	0/6	4/2	0.0303	yes
	0/6	7/2	0.00914	yes
ALVAC-*env,gag/prot*	2/4	4/2	0.28	no
	2/4	7/2	0.118	no
ALVAC-*97TMG*	3/3	4/2	0.5	no
	3/3	7/2	0.28	no
ALVAC-*env,gag/prot* &ICV	0/3	3/0	0.05	yes
	0/3	7/2	0.00914	yes

Discussion

The aim of this study was to evaluate the immunogenicity and protective efficacy of ALVAC-based FIV vaccines alone or in combination with ICV against experimental FIV challenge in cats. ALVAC recombinants tested in this study included ALVAC constructs encoding the FIV Env, the FIV Gag or both the FIV Env and Gag. Additionally, a recombinant was tested which encoded both the FIV Gag and a modified Env in which a putative immunosupressive region had been deleted.

We demonstrated that these recombinants were able to effectively infect non-permissive feline cells and express the inserted FIV genes. Upon inoculation in cats, ALVAC-specific humoral responses were readily detected. In contrast, FIV-specific humoral responses were detected only in cats that received a booster immunization with ICV. VN antibody titers were undetectable in all cats prior to challenge, even in those boosted with ICV. These observations are consistent with those of ALVAC-based HIV vaccine candidates in macaques, chimpanzees and humans in which humoral responses were weak or undetectable unless the animals received booster immunizations with subunit proteins (Franchini et al. 1995a; Abimiku et al. 1995; Piaoloux et al. 1995; Clements et al. 1996). Also, the induction of VN antibodies required boosting with HIV Env or peptides corresponding to the V3 region. Humoral responses including VN responses have been detected in chimpanzees immunized with ALVAC-HIV-1 recombinants alone (Van der Ryst et al. 1996; Girard et al. 1996). However, these responses were detected after a minimum of four immunizations only. Other FIV vaccine candidates composed of viral vectors including an attenuated adenovirus and a herpesvirus engineered to express the FIV Env, also failed to elicit FIV-specific humoral responses (Gonin et al. 1995; Verschoor et al. 1996).

The induction of humoral responses after the ICV boost is consistent with previous studies in our laboratory in which ICV vaccines were found to elicit FIV-specific humoral

responses but failed to induce VN antibody responses after a single immunization. Interestingly, cats immunized with ALVAC-*env,gag/prot* showed approximately a 10-fold higher immunoblot antibody titer than cats immunized with ALVAC vector alone after the ICV boost. Therefore it is possible that ALVAC-*env,gag/prot* induced FIV-specific T-helper responses resulting in a more efficient generation of FIV-specific humoral responses after exposure to ICV. This has been reported in other studies in which chimpanzees were immunized with an ALVAC recombinant encoding the HIV Env and boosted with recombinant Env. Chimpanzees immunized with ALVAC-Env produced antibodies following a single recombinant Env immunization, whereas chimpanzees immunized with recombinant Env alone failed to developed such antibodies after a single immunization (Girard et al. 1995). Similar priming of T-helper responses has also been shown in human volunteers immunized with ALVAC-HIV candidate vaccines (Piaoloux et al. 1995; Clements et al. 1996)(Tartaglia, personal communication)

The effectiveness of ALVAC-based vaccines in priming CTL responses as has been reported previously was further confirmed by findings in this study (Cox et al. 1993). Detectable levels of FIV-specific CTL responses were detected even after a single immunization. Although, the phenotype of the effector cells was not determined it was found that the effector cells reacted in a MHC-restricted manner. This finding excludes NK cells as the effector cell and implies a role for $CD8^+$ T-lymphocytes known to react in a MHC class I restricted manner. However, we can not exclude a role for $CD4^+$ CTL responses since the target cells (autologous PBMC) used in the assay could have presented FIV antigens in the context of both MHC class I and II. In an attempt to identify the FIV epitope recognized by these effector cells, CTL assays were performed using autologous target cells infected with vaccinia recombinants expressing FIV Env or Gag. These experiments did not demonstrate presence of CTL activity (data not shown), possibly due to technical difficulties of the CTL assay itself. The ability of ALVAC-based vaccines to prime CTL responses has been reported in several studies. Mice immunized with an

ALVAC-recombinant encoding the HIV-1 Env were shown to elicit CTL responses, including memory T-cell responses (Cox et al. 1993). The effector cells in these studies were characterized as $CD8^+$ T-lymphocytes. In addition, specific CTL responses mediated by $CD8^+$ T-lymphocytes were detected in 30% of the human volunteers immunized with an ALVAC recombinant encoding the HIV-1 Env (Pialoux et al. 1995; Egan et al. 1995). Interestingly, volunteers immunized with an ALVAC recombinant encoding both the HIV-1 Env and Gag, mounted CTL responses specific for Gag more often than for Env (Lawrence et al. 1996). We were unable to distinguish if this was the case in our study.

In summary, ALVAC-based FIV vaccines were found to differ from inactivated FIV-infected cell vaccines in their ability to elicit cell-mediated and humoral responses. This can be explained, in part, by a difference in the processing and presentation of ALVAC encoded immunogens. Immunogens encoded within ALVAC, require *de novo* expression within host cells to be presented to the host immune system. In this study, ALVAC immunizations were given intramuscularly and therefore it is likely that the majority of ALVAC infected muscle cells. These cells are capable of presenting immunogens in association with MHC class I and as such are expected to stimulate primarily CTL responses. Additionally, part of the ALVAC inoculum may have been taken up by macrophages or infected cells of the monocyte lineage, such as dendritic cells. These cells are capable of presenting antigens in association with both MHC class I and class II molecules and could therefore have stimulated the generation of both CTL and T-helper cell responses. This is supported by the low level of proliferative responses detected in some of the cats immunized with ALVAC-FIV recombinants alone. The direct stimulation of B-cell responses by ALVAC-encoded immunogens would require the expression and release of the immunogens from ALVAC-infected host cells. This process may have occurred at low level only, since ALVAC-FIV recombinants failed to induce detectable levels of humoral responses. Further, the nature of the immunogen itself may have played a role since ALVAC-recombinants expressing epitopes of viral pathogens other than retroviruses

have been shown to effectively elicit humoral responses including VN antibodies to the inserted immunogens (Taylor et al. 1992a, 1992b, 1991).

The protective efficacy ALVAC-based FIV vaccines was evaluated against 50 ID_{50} FIV_{Pet} (subtype A). This isolate differs only slightly (3% in Env and 1% in the Gag amino-acid coding region) from the $FIV_{Ville\ franche}$ isolate (subtype A) used to generate the ALVAC recombinants and is identical to the vaccine virus. After challenge, complete protection was obtained in all cats immunized with the ALVAC-*gag/prot* recombinant. The protection observed in this group correlated significantly with immunization as determined by the Fisher's exact test. In addition, virus was not detected in two naive SPF kittens transfused with cells obtained eight months postchallenge from ALVAC-*gag/prot* immunized cats whereas a kitten transfused with cells from an infected control cat became virus positive. Full protection was also observed in cats immunized with ALVAC-*env,gag/prot* and boosted with ICV. No significant protection was observed in cats immunized with ALVAC-*env*, ALVAC-*env,gag/prot*, and ALVAC-*97TMG* as determined by the Fisher's exact test ($P>0.05$). Important to emphasize at this point is the fact that we failed to obtain 100% infectivity in the control group immunized with ALVAC vector alone. Two out of six cats in this group tested virus negative by all criteria throughout the duration of the study. The infective dose (ID_{50}) of the virus challenge inoculum was titrated in cats obtained from a vendor different than the one used in this study. Therefore the true titer of the challenge inoculum may have been less than 50 ID_{50} explaining the lack of 100% infectivity in the control group. Further, lack of full infectivity could have been due to a general elevation of immune function by the ALVAC vector itself, although, no indication for such an elevation has been reported in any of the vaccine studies conducted with ALVAC. The inability to obtain full infectivity of control animals has also been observed in other FIV vaccine trials as well as SIV and HIV vaccine trials. In general, this is attributed to differences in genetic background which causes some animals to be more resistant to infection than others. For example, the HLA and HLA related genes have been

implemented in influencing the intensity and specificity of host immune-responses (Haynes et al. 1996). Also of some relevance to this issue are recent studies in which individuals with a homozygous defect in the CKR-5 loci encoding a coreceptor for HIV, were less likely found to become infected with HIV than individuals without this defect (Liu et al. 1996). Similar genetic based mechanism(s), yet to be determined, may be behind the observed variance in susceptibility to FIV infection in cats.

What constituted protective immunity in the ALVAC-*gag/prot* immunized cats is not clear. At the time of challenge and after challenge, the sera of these cats tested negative for Gag-specific and VN antibodies. Therefore, the presence of these antibodies did not appear to be crucial to the protection observed in this group. Moreover, vaccine trials in which cats were immunized with Gag proteins lacked protective efficacy despite the presence of Gag-specific antibodies. In fact, in these studies immunized cats showed enhanced infection. Thus, even if these responses would have been present they may not have played a role in the observed protection. However, we can not exclude a role for antibodies directed against Gag epitopes other than those tested for in the immunoblot and VN assays. Recent studies have demonstrated that HIV Gag proteins are displayed on the surface of infected cells (Ikatu et al. 1989; Shang et al. 1991). Similarly, cells infected with ALVAC-*gag/prot* may have expressed Gag on the host-cell surface and resulted in the induction of Gag-specific antibody dependent cell-mediated cytotoxic responses (ADCC). ADCC responses have been detected in HIV-infected individuals but these responses were predominantly directed against Env epitopes and attempts to demonstrate Gag-specific ADCC have failed thus far (Koup et al. 1988; O'Toole and Lowdell 1990). Protection in the ALVAC-*gag/prot*, may have been accomplished through Gag specific CTL responses. The induction of Gag-specific CTL responses has been reported in infected cats as well as cats immunized with ICV (Song et al. 1992; Flynn et al. 1995a). The importance of these responses in terms of protection, however, is still unknown. A vaccine based on a synthetic peptide containing epitopes of both the FIV Env and Gag was shown to

effectively induce Gag-specific CTL responses but failed to protect cats. No efficacy studies have been undertaken in monkeys to assess protection of ALVAC recombinants encoding Gag alone. Protocols in these models often involve priming and boosting with multiple antigenic determinants, making it difficult to resolve which epitope(s) are crucial for protection. However, there have been reports suggesting the importance of Gag epitopes in vaccine trials against other retroviruses. Vaccine efficacy of a herpes recombinant vector expressing FeLV Env was significantly enhanced with the inclusion of the Gag (Wardley et al. 1992). Furthermore, mice immunized with a vaccinia recombinant expressing the Gag of the Friend's murine leukemia retrovirus (F-MuLV) were protected against disease upon exposure to F-MuLV (Miyazawa et al. 1992).

Env-expressing ALVAC recombinants in our study lacked protective efficacy, similar to adenovirus and herpesvirus vectored vaccines encoding FIV Env (Gonin et al. 1995; Verschoor et al. 1996). Similar to ALVAC-*env*, these vector vaccines failed to induce Env-specific humoral responses including VN antibodies. The evaluation of cell-mediated responses was not included in these studies. In our study, ALVAC-*env* immunized cats were shown to elicit FIV-specific CTL responses. However, the presence of these responses did not correlate with protection. Chimpanzees immunized with an ALVAC-recombinant encoding the HIV-1 Env and boosted with recombinant Env (gp160) also failed to be protected. Lack of protection in these studies correlated with low VN antibody titers, since, chimpanzees immunized simultaneously with recombinant Env (gp160) and boosted with V3 peptide were protected in the presence of high VN antibody titers (Girard et al. 1995). Further, vaccine trials employing ICV and inactivated whole FIV in cats indicate that Env-specific humoral responses, including VN antibodies may be responsible for the observed protection (Yamamoto et al. 1991, 1993). Thus, the inability to generate such responses by the ALVAC-*env* vaccine could explain the lack of protective efficacy. Additionally, differences in the processing pathway, as discussed above, may

have resulted in immune responses against Env epitopes other than those generated upon immunization with ICV and inactivated whole virus.

The lack of protective immunity in the group immunized with ALVAC-*env,gag/prot* is somewhat surprising. It is possible that the inclusion of Env interfered with efficient presentation of Gag and as a result failed to properly prime the immune system. Immune responses directed against certain Env epitopes have been reported to cause enhancement of infection and as such could have negated the protective efficacy (Hosie et al. 1992; Siebelink et al. 1995; Osterhaus et al. 1996). This enhancement, however, was thought to be mediated by VN antibodies. In our study, VN antibodies were not detected prior to challenge. Further, virus isolation data did not show differences in the viral load among infected cats in the ALVAC-*env* group and infected cats in the control group immunized with ALVAC vector alone. Alternatively, a putative immunosupressive region in the transmembrane portion of Env could have interfered with the development of protective immune responses. However, cats immunized with the ALVAC-*97TMG*, a recombinant in which this region had been removed were readily infected at a same ratio as the ALVAC-*env,gag/prot* -immunized cats, implying that this region did not play a role. However, other immunosupressive regions located elsewhere than in the Env transmembrane region could have attributed to the reduction in immunogenicity of ALVAC-*env,gag/prot* recombinants. Similar to our findings, ALVAC recombinants expressing both the Env and Gag of HIV-2 failed to protect cynomolgus monkeys from HIV-2 challenge (Biberfeld et al. 1994). The challenge in this trial, however, was given by the mucosal route. Opposing our findings are studies in the chimpanzee model. Chimpanzees immunized with ALVAC recombinants expressing HIV-1 Env and Gag were protected from homologous HIV challenge (Van der Ryst et al. 1996; Girard et al. 1996). These animals, however, exhibited HIV-1-specific humoral responses including VN antibodies prior to challenge. Therefore, it would be of interest to determine if a higher dose of ALVAC-*env,gag/prot* or an increased number of immunizations could elicit such antibody responses in cats and enhance protective efficacy.

Interestingly, cats immunized with ALVAC-*env,gag/prot* but boosted with ICV were protected. The difference between this group and the ALVAC-*env,gag/prot* immunized group was the presence of FIV-specific humoral responses (antibodies detected by FIV immunoblot analysis) at the time of challenge. Control cats immunized with ALVAC vector alone and boosted once with ICV became infected despite the presence of FIV-specific antibody responses. The antibody titers in these control cats, however, were 10-fold lower than those detected in the ALVAC-*env,gag/prot*-primed cats. Based on this it could be speculated that priming with ALVAC-*env,gag/prot* enhanced the immunogenicity of ICV in a such a manner that protective immunity could be elicited after a single ICV immunization rather than after the three to four immunizations that are usually required to obtain protection. Interestingly, the protected cats in this group displayed low VN antibody titers shortly after challenge. These titers (5-20) differed significantly from those detected in infected control cats (>100). Therefore, VN antibody production may have been the result of anamnestic responses to the challenge inoculum. This is supported by the fact that VN antibodies in two of three cats could no longer be detected at 8 mo post-challenge. In the case of active infection, an increase rather than a decrease in VN antibody titers, is expected. The low level VN titer detected at 8 mo postchallenge in cat #PY4 presented a dilemma as this may have been the result of low level viral infection undetectable by RT and PCR analysis. Alternatively, this may have been the result of strong immunity that persisted which is more likely since this cat tested persistently negative by all other criteria up to one year postchallenge. Moreover, this cat was not able to resist superinfection with another FIV isolate (see chapter III) whereas two infected control cats resisted superinfection with this same FIV isolate. The generation of VN responses upon challenge in this group resembles observations in macaques which had been immunized with ALVAC recombinants encoding HIV-2 Env and Gag (Franchini et al. 1995a). After challenge, protected animals tested negative for virus but developed significant levels of viral neutralizing antibodies. Furthermore, the protective efficacy

obtained with ALVAC recombinants, in combination with protein based booster immunizations is similar to that observed in other animal models. Cynomolgus monkeys immunized with ALVAC-*env,gag/prot* (HIV-2) alone failed to be protected whereas those immunized with ALVAC-*env,gag/prot* and boosted with recombinant Env were partially protected from HIV-2 challenge (Biberfeld et al. 1994).

In summary, protective immunity can be obtained with ALVAC-based FIV vaccines encoding Gag or ALVAC-based FIV vaccines encoding Env and Gag in combination with ICV. This is the first study to report the induction of protective immunity against experimental FIV challenge in cats utilizing a viral vector-based vaccine. Protection obtained with ALVAC-based vaccines encoding Gag may have occurred via cell-mediated responses including CTL and ADCC, or other mechanisms. Protection in ALVAC-*env,gag/prot* and ICV immunized cats may have been mediated by these same mechanisms. Additionally, humoral responses including low but significant VN antibody titers may have contributed to the protection observed in this group.

CHAPTER III
EFFICACY EVALUATION OF CANARYPOXVIRUS (ALVAC)- BASED FIV VACCINE COMBINED WITH INACTIVATED FIV-CELL VACCINE AGAINST HETEROLOGOUS FIV CHALLENGE IN CATS

Introduction

Based on genetic variation in the Env and Gag coding regions, HIV isolates obtained worldwide have been classified into several subtypes or clades (Cheingsong-Popov et al. 1994; Brodine et al. 1995). Optimally, a vaccine against HIV should induce immune responses that can cross react with a wide variety of these HIV subtypes. Thus far, HIV vaccine trials have concentrated mainly on a single subtype, subtype B, as this represents the predominant type found in Europe and the United States. However, the emergence of HIV isolates other than subtype B is increasing significantly. Furthermore, 90% of the reported HIV cases are in the developing countries where the HIV epidemic encompasses multiple subtypes. These countries in particular would benefit from the development of broad spectrum vaccines since drug treatments, even if they would become available, would be too expensive.

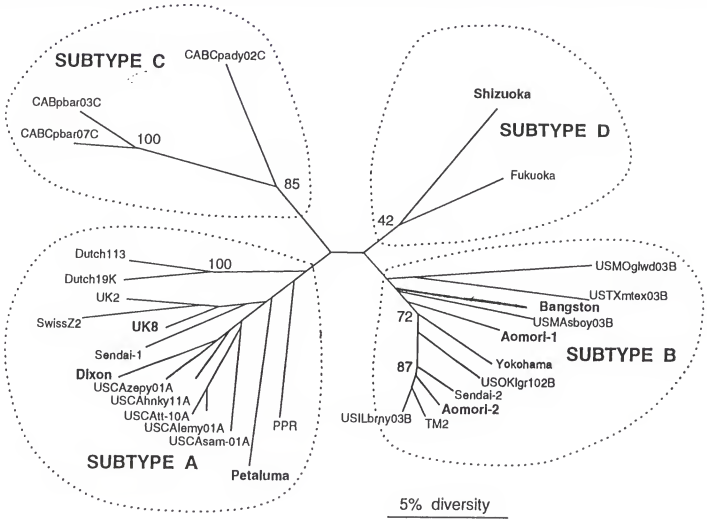
Similar to HIV, FIV strains have been classified into different subtypes (A-D), based on aa sequence differences in Env and to a lesser extent in the Gag (Sodora et al. 1994; Kakinuma et al. 1995; Rigby et al. 1993) (Figure 3.1). As such, the FIV model provides a means to assess the protective efficacy of vaccine strategies against multiple HIV subtypes. Vaccine protection against homologous and slightly heterologous FIV strains (within one subtype) has been achieved with inactivated whole virus and inactivated FIV-infected cell vaccines (ICV) (Yamamoto et al. 1991, 1993; Verschoor et al. 1995; Johnson et al. 1994; Hosie et al. 1995). These same vaccines,

however, failed to induce protective immunity against distinctly heterologous FIV strains from heterologous subtypes (Yamamoto et al. 1993; Johnson et al. 1994; Hosie et al. 1995). Thus, a modified or different vaccine approach is required to induce immune responses that will cross-react with a wide variety of FIV subtypes.

Cross-protective immunity has been obtained against heterologous HIV-1 infection in chimpanzees using vaccines composed of whole Env or Env fragments from multiple isolates (Girard et al. 1995). However, the use of viral vector-based vaccines may overcome the need for the inclusion of antigenic determinants from multiple isolates. Viral vector-based vaccines have been shown to effectively prime cell-mediated responses whereas conventional vaccines have been shown to prime predominantly humoral responses. The epitopes recognized by cell-mediated responses may be directed against epitopes that are more conserved among different isolates and as such provide protection against a wider variety of HIV isolates. In fact, preliminary findings from vaccine trials in which macaques were immunized with a canarypoxvirus (ALVAC) vectored HIV-1 vaccines suggest that this may be the case (Abimiku et al. 1995). After immunization these macaques were partially protected from infection with a distinctly heterologous HIV-2 isolate.

In the previous chapter, we demonstrated that cats immunized with ALVAC-recombinants encoding FIV Env and Gag (ALVAC-*env,gag/prot*) and boosted with a conventional inactivated FIV-infected cell vaccine (ICV), resisted infection with FIV Petaluma (FIV_{Pet}), a subtype A isolate. This isolate was closely related to the isolate used to generate the ALVAC-FIV recombinant ($FIV_{Ville\ franche}$, subtype A) and identical to the FIV isolate used to generate the ICV vaccine. In this study, we evaluated if these ALVAC-*env,gag/prot*/ICV immunized cats could be protected from a second challenge with a distinctly heterologous FIV isolate, FIV Bangston (FIV_{Bang}). The FIV_{Bang} isolate is classified as a subtype B virus and differs from the FIV_{Pet} isolate (subtype A) by 21% in the Env and 2.4% in the Gag amino-acid sequence.

Figure 3.1. Phylogenetic relationship between FIV-isolates comparing envelope sequences. The four subtypes (A-D) are grouped with circles.



Materials and Methods

Animals and grouping

Eight cats were used in this study including three ALVAC-*env,gag/prot*/ICV immunized cats (#PY4, #QH3, #QA6) and two FIV_{Pet} infected control cats (#PY2 and #QE4) (see Chapter II, Materials and Methods). Also included were three age matched SPF cats (#EJ2, #DH3, #GU5), purchased from Liberty Research Inc., which received no immunizations prior to the FIV_{Bang} challenge.

Challenge inoculum

The challenge inoculum consisted of cell-free culture fluid from PBMC infected with FIV_{Bang} previously titered in SPF cats. The challenge inoculum of 75 ID_{50} was given i.p. 8 months after the initial FIV_{Pet} challenge.

Viral status monitoring

Viral infection was monitored by RT activity and FIV-specific PCR and by evaluating the level of FIV-specific humoral responses, including viral neutralizing (VN) antibody responses to both the FIV_{Pet} and FIV_{Bang} isolate, as described previous (see Chapter II, Materials and Methods).

DNA sequencing

Nucleotide sequencing of the amplified PCR products was performed as described previously using FIV specific primers: FIV_{Pet} (5'-TAGTAGTTATAGTGGTACTA-'3) and FIV_{Bang} (5'-GGGACTACTAGCAATGGAATA-'3) (see Chapter II, Materials and Methods).

Results

In this study, ALVAC-*env,gag/prot*/ICV immunized cats, which were previously shown to resist challenge with FIV_{Pet} a homologous subtype strain (subtype A), were given a second challenge with 75 ID_{50} FIV_{Bang}, a subtype B virus. Additionally, two FIV_{Pet}-infected control cats were challenged with FIV_{Bang} to determine if active infection could prevent superinfection with the FIV_{Bang} isolate. As presented in Table 3.1, all nonimmunized/noninfected control cats became readily viremic after FIV_{Bang} challenge. In contrast, ALVAC-*env,gag/prot*/ICV immunized cat #QA6 remained virus negative as determined by virus isolation (RT) and PCR in peripheral blood up to four months postchallenge. Cats #PY4 and #QH3 remained virus negative as determined by virus isolation (RT) in peripheral blood but tested positive by PCR at three months and four months postchallenge, respectively. Nucleotide sequence analysis of the amplified PCR product from PBMC revealed FIV_{Bang}-specific sequences only (data not shown). The two FIV_{Pet} -infected control cats (#QE4 and #PY2) tested positive by RT and PCR before and after the FIV_{Bang} challenge. However, nucleotide sequence analysis of the FIV-specific PCR products obtained from these cats only verified FIV_{Pet}-specific sequences up to 4 months after the FIV_{Bang} challenge (data not shown).

Prior to challenge, FIV-specific antibodies, as determined by immunoblot, were present in all ALVAC-*env,gag/prot*/ICV immunized cats. Similarly, sera from FIV_{Pet} -infected control cats (#PY2 and #QE4) contained FIV-specific antibodies which were slightly higher in titer than those detected in the ALVAC-*env,gag/prot*/ICV immunized group (Table 3.1). As expected, sera from the naive control cats (#EJ2, #DH3, #GU5) did not contain detectable levels of FIV-specific antibodies before the FIV_{Bang} challenge (Table 3.1). Viral neutralizing antibodies to either FIV_{Pet} or FIV_{Bang} were absent in all naive control cats and in two of three ALVAC-*env,gag/prot*/ICV-immunized cats (#QA6, #QH3). In contrast, sera of the FIV_{Pet}-infected control cats (#PY2 and #QE4) had high

Table 3.1 Immune parameters and viral status before and after FIV_{Bang} challenge.

Cat #	Vaccine	pre-2nd-challenge[a] WB	VN_{Pet}	VN_{Bang}	post-2nd-challenge[b] WB	VN_{Pet}	Vn_{Bang}	virus status RT/PCR (wks p.c.) 2	6	10	14	18
QA6	ALVAC-*env,gag/prot* & ICV	+(4-5)	<5	<5	+	5-20	<5	-/-	-/-	-/-	-/-	-/-
PY4	ALVAC-*env,gag/prot* & ICV	+(4-5)	5-20	5-20	+	5-20	<5	-/-	-/-	-/-	+[c]/+	-/+
QH3	ALVAC-*env,gag/prot* & ICV	+(4)	<5	<5	+	5-20	<5	-/-	-/-	-/-	-/-	-/+
EJ2	-	-	ND	<5	+	ND	<5	-/-	+/+	+/+	+/+	NT
GU5	-	-	ND	<5	+	ND	<5	-/-	+/+	+/+	+/+	NT
DH3	-	-	ND	<5	+	ND	>100	-/-	+/+	+/+	+/+	+/+
PY2	ALVAC	+(5-6)	>100	<5	+	>100	<5	+/+	-/-	+/+	+/+	-/+
QE4	ALVAC&ICV	+(5-6)	>100	<5	+	>100	<5	+/+	-/+	+/+	+/+	+/+

[a] serum samples taken at the day of challenge.
[b] serum samples taken 3-4 months post-2nd-challenge.
[c] weakly positive by RT (2X background cpm)
ND= not determined
NT= not tested, animal euthanized.

VN antibody titers (>100). More importantly, these responses were specific for FIV_{Pet} and did not cross-neutralize FIV_{Bang} *in vitro*. Interestingly, serum taken before the FIV_{Bang} challenge from cat #PY4 contained low level VN antibodies(<20) which neutralized both FIV_{Pet} and FIV_{Bang} *in vitro*.

After challenge, all naive control cats developed FIV-specific antibodies as determined by immunoblotting. In contrast, FIV_{Bang}-specific VN antibodies were detected in only one of the three cats (#DH3). Interestingly, all three cats immunized with ALVAC-*env,gag/prot*/ICV developed low level of VN antibodies to FIV_{Pet} similar to that observed after the initial FIV_{Pet} challenge but failed to develop VN antibodies specific for FIV_{Bang}. Similarly, FIV_{Pet}-infected control cats failed to develop FIV_{Bang}-specific VN antibodies after challenge whereas FIV_{Pet}-specific antibodies persisted at high titers (>100).

Discussion

In this study, cats immunized with both ALVAC-*env,gag/prot* and ICV were partially protected from heterologous FIV subtype challenge given eight months after an initial homologous FIV challenge. These data should be interpreted with some caution as they reflect only a 4 month period postchallenge. Nevertheless, immunized cats showed a delay in infection, as all control cats became viremic within 6 weeks postchallenge whereas two of the immunized cats became positive based on PCR analysis only at 14 and 18 weeks postchallenge, respectively. Furthermore, throughout the study these cats were negative for virus isolation, demonstrating a significant reduction in viral load.

The exact immune-mechanism(s) responsible for the delay in infection or the reduction in viral load in the ALVAC-*env,gag/prot*/ICV immunized cats is unclear. It is possible that FIV-specific humoral responses present prior to the FIV_{Bang} challenge played a role. Both the ALVAC-*env,gag/prot*/ICV immunized cats and FIV_{Pet}-infected control

cats had high titer of FIV-specific humoral responses prior to challenge whereas the naive control cats lacked such responses. On the other hand, ICV vaccines capable of eliciting high titers of FIV-specific antibody responses were effective against homologous and slightly heterologous challenges but not against distinctly heterologous FIV isolates (Yamamoto et al. 1991, 1993; Johnson et al. 1994). It is possible that the presentation of both exogenous and endogenous FIV antigens in the ALVAC-*env,gag/prot*/ICV immunized cats resulted in the generation of higher titers of antibody responses or antibodies to a wide variety of FIV epitopes including those shared by FIV_{Pet} and FIV_{Bang} isolates. Similarly, active FIV_{Pet} infection in the infected control cats could have broadened humoral responses, resulting in resistance to superinfection. This is consistent with results from a previous study in which a long-term FIV_{Bang}-infected cat was protected from FIV_{Pet} infection in the presence of high titer FIV-specific antibody responses. Likewise, cross-protection by infection with HIV-2 in high risk woman has been found to correlated with resistance to infection with HIV-1 which is genetically highly divergent from HIV-2 (Kanki et al. 1995). Additionally, monkeys infected with an attenuated macrophage tropic SIV strain were resistant to superinfection with a highly virulent SIV strain displaying 16% difference in the Env amino-acid sequence (Clements et al. 1995). In these studies, the length of infection and the concomitant broadening of humoral immune responses positively correlated with protection. Moreover, naive monkeys passively immunized with serum from these long-term infected monkeys were partially protected from subsequent heterologous challenge. The broadening of humoral responses in these monkeys, however, also included the broadening of VN antibody responses capable of cross-reacting with the challenge inoculum virus. In contrast, chimpanzees immunized with recombinant Env and V3 peptides corresponding to two HIV-1 isolates, were protected from heterologous subtype challenge in the presence of VN antibodies that neutralized vaccine strains but not the challenge strain (Girard et al. 1995).

In our studies, cross-reactive VN antibody responses may not have played a key role in vaccine protection. Prior to the FIV_{Bang} challenge, VN antibody titers were detected in both FIV_{Pet}-infected control cats and in the ALVAC-*env,gag/prot*/ICV-immunized cat #PY4. Except for the low VN antibody titer detected in cat #PY4, these VN antibody responses were specific for FIV_{Pet} and did not cross-react with FIV_{Bang} Surprisingly, cat #PY4 was the first one of the ALVAC-*env,gag/prot*/ICV immunized group to become positive by PCR analysis, suggesting that the presence of FIV_{Bang}-specific VN antibodies prior to challenge was not beneficial. Again, this resembles the findings from superinfection studies in which FIV_{Bang}-infected cats which developed FIV_{Bang}-specific antibody responses were susceptible to superinfection FIV_{Pet} whereas those that lacked FIV_{Bang}-specific VN antibodies resisted superinfection (Okada et al. 1994). Furthermore, cats #QA6 and #QH3 showed a delay in infection (i.e. partial protection) without detectable VN antibodies at the time of challenge, suggesting that these antibodies were not responsible for this delay. However, the low titers of VN antibodies detected after challenge, similar to those observed after the initial FIV_{Pet} challenge may have contributed, although the mechanisms behind this remains to be identified.

In addition to humoral immunity, FIV-specific CTL activity may have contributed to the delay in infection and/or reduction in viral load. In the previous chapter, the presence of FIV_{Pet}-specific CTL activity was reported in the ALVAC-*env,gag/prot* /ICV immunized cats. Unfortunately, cross reactivity to FIV_{Bang} was not evaluated due to limited amount of PBMC that could be harvested from these cats. However, the generation of cross-reactive CTL responses against FIV_{Bang} has been detected in cats long-term infected with the FIV_{Pet} (unpublished data). Thus, ALVAC-*env,gag/prot* and ICV immunizations could have elicited similar cross-reactive CTL responses. These responses may have been directed against Gag epitopes in particular, since the amino acid sequence difference between the FIV_{Pet} and FIV_{Bang} Gag protein is significantly less

(approx. 2.4 %) than the difference displayed at the Env (approx. 21 %). These findings resemble those of ALVAC-HIV-1 trials in macaques (Abimiku et al. 1995). Macaques immunized with ALVAC-HIV-1 recombinants and boosted with subunit proteins were partially protected and had a delay in infection after challenge with distinctly heterologous HIV-2. Prior to challenge these macaques exhibited HIV-1 specific cell-mediated responses and VN responses that effectively prevented HIV-1 infection but failed to prevent HIV-2 infection *in vitro*.

In summary, prime/boost protocols involving priming with ALVAC-FIV recombinants followed by boosting with inactivated FIV-infected cells vaccines can elicit partial protection or delay in infection of distinctly heterologous FIV isolates from heterologous subtypes. The exact immune-correlates of protection are unclear. Although, the findings in our study suggest a role for both cell mediated and humoral responses.

CHAPTER IV
SUMMARY AND FUTURE STUDIES

Synopsis

Comparative studies on the efficacy and immunogenicity of various ALVAC-based FIV vaccines alone or in combination with inactivated FIV-infected cell vaccines support the following conclusions:

(1) ALVAC-based FIV vaccines are ineffective in priming B-cell responses but effective in priming cytotoxic T-cell responses and low level T-helper responses specific for the inserted FIV antigens.

(2) A booster immunization with ICV following immunization with ALVAC-based FIV vaccines enhances immunogenicity as determined by induction of detectable FIV-specific humoral responses.

(3) ALVAC-based FIV vaccines encoding the FIV Gag and Prot can induce protective immunity against experimental challenge with slightly heterologous FIV isolates.

(4) ALVAC-based FIV vaccines combined with ICV can induce protective immunity against a slightly heterologous FIV isolate and delay infection with distinctly heterologous FIV isolates of other subtypes.

The data obtained in this study were generated from a relatively small study group. Future studies, should include larger numbers of animals to add to the statistical significance of the obtained data. Further, the duration of the ALVAC-FIV-induced protective immunity and the efficacy against various routes of challenge, in particular the

mucosal route, should be evaluated. In addition, the protective efficacy should be examined against FIV field isolates, higher challenge doses and other types of challenge inocula, such as cell-associated and plasma-derived virus. Moreover, studies should be undertaken to further assess the protective efficacy against FIV strains of heterologous subtypes. It will be particularly interesting to evaluate if cats immunized with ALVAC-*gag/prot* alone can resist heterologous subtype challenge since sequence variability in the *gag* gene is significantly lower between different FIV isolates when compared to the variability observed in the *env* gene. Therefore, protective immune responses directed against Gag epitopes may cross-react with a wider range of FIV isolates. If such protection can not be achieved, ALVAC recombinants encoding Gag of multiple FIV isolates could be tested for their ability to elicit broad-range protective immunity.

Furthermore, it would be of interest to evaluate immunization protocols involving priming with ALVAC-*gag/prot* in combination with inactivated FIV-infected cell vaccine. This may be a more optimal combination than ALVAC-*env,gag/prot* in combination with ICV as ALVAC-*env,gag/prot* alone lacked protective efficacy.

Although we were able to demonstrate protective immunity upon immunization with ALVAC-based FIV vaccines, the mechanism(s) involved in the observed protection remains unclear. This important issue needs to be addressed in future studies. To further delineate the role of cell mediated and humoral responses naive cats could be passively immunized with serum or cells from ALVAC-FIV immunized cats and subsequently tested for their ability to resist experimental FIV infection. Further, efforts should be made to improve the current methods used to assess the induction of cell-mediated responses in particular CTL responses. At present, these assays require large numbers of cells and prolonged *in vitro* culturing which may result in inaccurate representation of what actually occurs *in vivo*. Since our study implies a role for Gag in protection the next step would be

to evaluate which of the Gag proteins, CA, NC or MA are required to induce protective immunity. Particular attention should be paid to the Gag p17 protein as vaccine studies involving HIV-1 p17 Gag protein have demonstrated both the induction of CTL, T-help and VN antibody responses. Moreover, SCID mice transfused with PBMC from human volunteers immunized with a synthetic p17 were reported to be protected from HIV-1 challenge whereas control mice transfused with cells from nonimmunized subjects became infected (Goldstein et al. 1993).

Overall, these studies should add to our understanding of the interactions between lentiviral pathogens and the host immune system and aid in the development of safe and effective vaccines against FIV as well as HIV. Additionally, these studies may aid in the development of vaccines against other pathogens that display continuous antigenic variation and pathogens for which current vaccine approaches have failed.

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BIOGRAPHICAL SKETCH

Marinka Tellier was born in the summer of 1968 and grew up on the islands surrounded by the salty waters of the "Noordzee" and the "Schelde" in the Netherlands. At the age of 4 she was first sent to school by her parents not knowing that this episode would continue for the next 24 years of her life. In the first eight she visited the Prinsenhoven School in Middelburg. She then went to the Christelijke Scholengemeenschap Walcheren from which she graduated in 1986. She continued her education at the College of Pharmacy at the State University of Utrecht in the Netherlands. After enjoying student life for a period of five years she graduated with a M.S. degree in pharmacy in 1991.

In the summer of 1993, she started her graduate work under the supervision of Dr. Yamamoto with no experience in the feline field. In fact, at the time she did not even realize there was such a thing as feline immunodeficiency virus. In the last three years she has become impressed by this little creature that encompasses a little more than 9000 basepairs. Now, a little wiser and a little more confused, she will go on and keep on wondering if our curiosity will do to us what it did to the cat.